Effect of Persistent Antigen Expression on Cytomegalovirus-Specific T Cell Responses.

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Abstract

More than 90% of the human population is latently infected with the Cytomegalovirus (CMV). Infectious virus is not produced during latent infection, but the viral genomes remain in infected host cells for years and retain the ability to reactivate upon episodes of immune suppression. The murine CMV (MCMV) and the human CMV (HCMV) infection share important similarities in terms of pathogenesis and immunity and MCMV is one of the best characterised and most commonly used *in vivo* models of CMV infections. The immediate-early genes *ie1* and *ie3* from MCMV are homologues of *ie1* and *ie2* from HCMV. These genes play critical regulatory roles in primary infection and in reactivation from latency in both viruses. During viral latency, the immune system is weakly, but constantly, stimulated by viral antigens, resulting in an expansion of CMV-specific $CD8^+$ T cells, a phenomenon termed Memory Inflation (MI). The large fraction of CMV-specific $CD8^+$ T cells is composed of effector memory T cells (T_{EM}), and it is assumed that these cells are short-lived, but regularly replenished with antigen specific cells from other T cell subsets.

To define whether expression of the *ie1* and *ie3* genes during latency is responsible for maintenance of MI, the recombinant virus MCMV IE1/3^{flox} was generated by flanking the *ie1/3* transcriptional unit with *loxP* sites, which allows the recombination of the target sequence by the enzyme Cre recombinase. The virus was tested in a newly established *in vitro* latency model in which the addition of IFNβ reversibly inhibits MCMV replication. Upon IFNβ retraction, MCMV WT, but not MCMV IE1/3^{flox}, reactivated from cells expressing the Cre recombinase, indicating that Cre excised the *ie1/3* region in the presence of IFNβ-recruited cellular repressors. To test the system *in vivo*, mice expressing a Tamoxifen (Tam)-inducible Cre recombinase (Cre.ER^{T2}) were infected with MCMV IE1/3^{flox} or MCMV WT. In the absence of Tam, non-activated Cre.ER^{T2} did not influence the MI kinetic. The administration of Tam at 4 months post infection should have allowed the selective targeting of the *lox*P sites at times of viral latency. Indeed, the percentage of MCMV-specific inflationary $CD8^+$ T cells and the relative size of the T_{EM} cell subset were transiently decreased upon Tam administration in Cre.ER^{T2} mice, but not in the parental C57BL/6 strain. However, unexpectedly, activated Cre.ER^{T2} had the same effect in mice infected with MCMV WT, strongly arguing that the effects were not due to the targeted recombination of viral genomes. This apparent contradiction could be explained by direct toxicity of Cre.ER^{T2} on the proliferating T cells. Analysis of the $CD8^+$ T cell subsets in the spleens of latently infected Cre.ER^{T2} mice for their susceptibility to Cre-mediated toxicity revealed

increased apoptosis only in the T_{EM} cell subset, whereas naive T cells and central memory T cells (T_{CM}) were not affected. This suggests that the observed reduction of T_{EM} cells was not caused by the elimination of cycling naive or T_{CM} cells and consequently less recruitment of these cells to the T_{EM} cell subset, because only the T_{EM} cells were directly affected by Tam administration to Cre.ER^{T2} mice. In conclusion, the results argue that inflationary $CD8^+$ T cells and T_{EM} cells in general are maintained, at least in part, by cyclic T_{EM} cells.

Zusammenfassung

Mehr als 90% der menschlichen Bevölkerung sind latent mit dem Zytomegalievirus (eng. Cytomegalovirus (CMV)) infiziert. In dieser Phase der Infektion werden keine infektiösen Viren produziert, aber deren Genome lassen sich in infizierten Wirtszellen nachweisen. In Folge von Immunsuppressionen kann das Virus reaktivieren. Die murine CMV (MCMV) und die menschliche CMV (HCMV) Infektion haben wichtige Gemeinsamkeiten in Bezug auf die Pathogenese und Immunität, wobei MCMV eines der am besten charakterisierten und meistgenutzten *in vivo* Modelle der CMV-Infektion ist. Die „immediate-early" Gene *ie1* und *ie3* von MCMV sind Homologe der Gene *ie1* und *ie2* von HCMV. In beiden Viren haben diese Gene kritische regulatorische Rollen während der primären Infektion und der Reaktivierung aus der Latenz. Während der viralen Latenz wird das Immunsystem zwar nur gering, aber permanent durch virale Antigene stimuliert, wodurch es zu einer Expansion von CMV-spezifischen T-Zellen kommt, ein Phänomen, welches als „Memory Inflation" (MI) bezeichnet wird. Der Großteil der CMV-spezifischen $CD8^+$ T-Zellen besteht aus Effektorgedächtniszellen (T_{EM}-Zellen). Es wird angenommen, dass diese Zellen kurzlebig sind und regelmäßig von Antigen-spezifischen Zellen anderer T-Zellsubpopulationen ersetzt werden.

Um zu klären, ob die Expression der Gene *ie1* und *ie3* während der Latenz für die Aufrechterhaltung der MI verantwortlich ist, wurde das rekombinante Virus MCMV IE1/3^{flox} erschaffen. Bei diesem wurde die Transkriptionseinheit von *ie1/3* mit *loxP*-Stellen flankiert, was die Rekombination der dazwischenliegenden Sequenz durch das Enzym Cre-Rekombinase erlaubt. Das Virus wurde in einem neu entwickelten *in vitro* Latenzmodell getestet, in dem die Zugabe von IFNß die Replikation von MCMV reversibel inhibiert. Nach dem IFNβ-Entzug reaktivierte MCMV WT, aber nicht MCMV IE1/3^{flox}, in Zellen welche die Cre-Rekombinase exprimieren. Dies weist darauf hin, dass das Enzym in Gegenwart von IFNß-rekrutierten zellulären Repressoren die Gene *ie1* und *ie3* entfernen konnte. Um das System *in vivo* zu testen, wurden C57BL/6 Mäuse, welche eine Tamoxifen (Tam)-induzierbare Cre-Rekombinase (Cre.ER^{T2}) exprimieren, mit MCMV IE1/3^{flox} oder MCMV WT infiziert. In Abwesenheit von Tam, hatte die inaktive Cre.ER^{T2} keinen Einfluss auf die MI. Die Verabreichung von Tam, 4 Monate nach der Infektion, sollte erreichen, dass das Enzym erst während der viralen Latenz Zugriff auf die *loxP*-Stellen hätte. Tatsächlich wurde der Anteil der inflationären MCMV-spezifischen $CD8^+$ T-Zellen und der relative Anteil der T_{EM}-Zellen nach der Verabreichung von Tam in Cre.ER^{T2} Mäusen vorübergehend reduziert. Allerdings erzeugte die aktivierte Cre.ER^{T2} diesen Effekt unerwarteter Weise auch bei Mäusen,

welche mit MCMV WT infiziert waren, während dies nicht in normalen C57BL/6 Mäusen beobachtet werden konnte. Dies lässt darauf schließen, dass der Effekt nicht auf die Rekombination des viralen Genoms zurückzuführen ist. Dieser scheinbare Widerspruch könnte durch eine direkte Toxizität der Cre.ER^{T2} auf proliferierende Gedächtnis-T-Zellen erklärt werden. Die Analyse verschiedener CD8$^+$ T-Zellsubpopulationen auf deren Anfälligkeit für Cre-vermittelte Toxizität aus der Milz latent infizierter Cre.ER^{T2} Mäuse, ergab einen höheren Anteil von apoptotischen Zellen ausschließlich in der T_{EM}-Zellsubpopulation, während die naiven T-Zellen und die zentralen Gedächtnis-T-Zellen (T_{CM}) nicht betroffen waren. Dies deutet darauf hin, dass die beobachtete Abnahme von T_{EM}-Zellen nicht durch die Eliminierung proliferierender naiver T-Zellen oder T_{CM}-Zellen verursacht wurde, was zu weniger Rekrutierung dieser Zellen zur T_{EM}-Zellsubpopulation führen würde, weil ausschließlich die T_{EM}-Zellen direkt von der Verabreichung von Tam beeinflusst worden zu sein schienen. Daraus lässt sich schließen, dass die Anzahl von inflationären CD8$^+$ T-Zellen, und T_{EM}-Zellen im Allgemeinen, zumindest teilweise, durch zyklische T_{EM}-Zellen aufrechterhalten bleibt.

Abbreviations

7AAD	7-Amino-Actinomycin D
Ab	antibody
ACK	Ammonium-Chloride-Potassium
AIDS	acquired immune deficiency syndrome
Amp	Ampicillin
AP-1	activator protein 1
APC	antigen presenting cell
BAC	bacterial artificial chromosome
bp	base pair
C	capacity
℃	degree Celsius
CAM	Chloramphenicol
CBF1/RBP-Jκ	centromere-binding factor 1/recombination signal binding protein J kappa
CD	clusters of differentiation
cDC	conventional dendritic cell
CIR	corepressor interacting with RBP J
cm	centimetre
CMV	Cytomegalovirus
CO_2	carbon dioxide
CompBeads	compensation beads
Cre	causes recombination
Cre-ER	Cre-oestrogen receptor
CTL	cytotoxic T lymphocyte
Daxx	death-domain associated protein
DC	dendritic cell
dH_2O	deionised water
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOG	deoxy-Galactose
Dox	Doxycycline
dpi	days post infection
EC	endothelial cell

E. coli	*Escherichia coli*
ER	endoplasmatic reticulum
ER^T/ER^{T2}	modified oestrogen receptor
EtOH	ethanol
FACS	fluorescence activated cell sorting
FCM	flow cytometer
FCS	fetal calf serum
g	gravity of earth
galK	galactokinase
gB	glycoprotein B
GOI	gene of interest
h	hour
H_2O	water
HBSS	Hank's balanced salt solution
HCMV	human Cytomegalovirus
HDAC	histone deacetylases
HSV-1	Herpes Simplex Virus 1
HZI	Helmholtz-Zentrum für Infektionsforschung
ICCS	intra cellular cytokine staining
ie	immediate-early
IFN	Interferon
IFN-I	type I Interferon
IFN-II	type II Interferon
Ig	Immunoglobulin
IL	Interleukin
i.p.	intraperitoneal
Kn	Kanamycin
kb	kilo base
kV	kilo Volt
LB	Luria Bertani
loxP	locus of crossing over (x), P1
LSECs	liver sinusoidal endothelial cells
M	Molar
MCMV	murine Cytomegalovirus
MI	memory inflation
MIE	major immediate-early enhancer
MIEP	major immediate-early enhancer promoter

MEFs	mouse embryonic fibroblasts
MHC	major histocompatibility complex
MHC-I	major histocompatibility complex class I
MHC-II	major histocompatibility complex class II
µF	micro Farad
µg	micro grams
µl	micro litre
µM	micro Molar
mg	milligrams
ml	millilitre
mM	milli Molar
min	minute
MOI	multiplicity of infection
mRNA	messenger RNA
msec	millisecond
N	Nitrogen
ND10	nuclear domain 10
NFκB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanograms
NK cell	natural killer cell
nm	nanometer
O_2	Oxygen
OD	optical density
ORF	open reading frame
ON	over night
PBS	phosphate buffered saline
PCR	Polymerase chain Reaction
pDC	plasmacytoid dendritic cell
pfu	plaque forming unit
p.i.	post infection
pmol	pikomol
R	Resistance
R26	Rosa26
RAG-1/-2	recombination-activating gene 1/2
rpm	revolutions per minute
RPMI	Roswell Park Memorial Institute medium
RNA	ribonucleic acid

RT	room temperature
rtTA	reverse tetracycline-controlled transactivator
SD	standard deviation
sec	second
SEM	standard error of the mean
SN	supernatant
STAT	signal transducer and activator of transcription
Suppl. DMEM	DMEM supplemented with 10% FCS, 1% Glutamine and 1% Penicillin/Streptomycin
TAP	transporter associated with antigen processing
T_{CM}	central memory T cell
TCR	T cell receptor
T_{EM}	effector memory T cell
tetO	operator sequences or tetracycline-responsive elements
TNF	tumour-necrosis-factor
U	electric tension
UV	ultraviolet
V	Volt
VSB	virus standard buffer
WT	wild type
YY1	Yin Yang 1
Ω	Ohm

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1 Introduction

1.1 Herpesvirus

Cytomegaloviruses (CMVs) are members of the large family *Herpesviridae* (*gr. herpein*, to creep). These viruses belong to the *Herpesvirales* which are part of the double stranded DNA viruses (according to the International Committee on Taxonomy of Viruses (ICTV)).

According to their replicative cycle and host range, *Herpesviridae* are classified into three subfamilies: *alphaherpesvirinae*, *betaherpesvirina*e and *gammaherpesvirinae*. *Herpesviridae* members have in common that they are able to establish a life-long latent infection. This means, that viral genomes persist in the organism in the absence of infectious viral particles, after the primary infection has been cleared. The remaining genomes are silenced in a reversible manner, which may lead to viral recurrence if the host immune system becomes weakened and cannot control the virus anymore. Herpesvirus infections were identified in almost all vertebrate classes, including mammals, birds, reptiles, amphibians or fishes (reviewed in [1]), but also in invertebrates, for example in some bivalve species [2].

All members of the *Herpesviridae* family are large enveloped viruses, e.g. the human CMV (HCMV), a *betaherpesvirina*e, has a size of 150 – 200 nm. The DNA is packed, together with some proteins, in an icosahedral nucleocapsid and the capsid is surrounded by a dense proteinaceous matrix: the tegument. This in turn is enveloped by a host-derived lipid bi-layer which is decorated with viral glycoproteins (Figure 1) (reviewed in [3]).

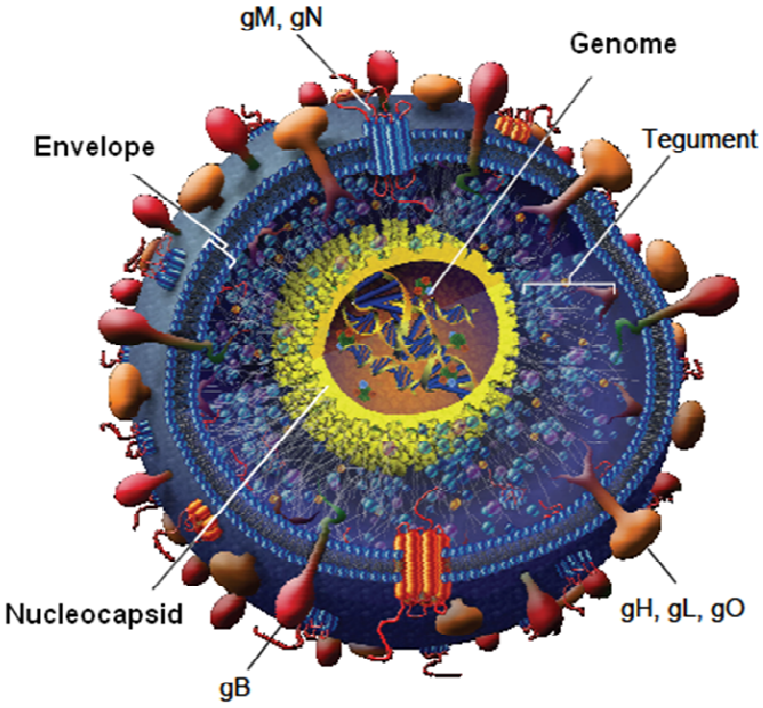

Figure 1 Structure of HCMV virion.

Herpesvirus virions are composed of three layers. The first inner layer consists of the nucleocapsid, which contains the double-stranded viral DNA genome. The capsid is surrounded by the tegument. The tegument is enveloped by a host-derived lipid bi-layer which is studded with viral glycoproteins. (modified from Streblow *et a,* [4])

1.2 Cytomegalovirus

CMVs belong to the *betaherpesvirina*e, characterised by slower reproduction than the members of the other two subfamilies. The name cytomegalovirus comes from the Greek *cyto*, which means “cell” and *megalo*, which means “large”. CMVs are strictly species-specific, it can only replicate in cells of the host it coevolved with or in cells of a closely related species (reviewed in [5]) [6-9]. Due to this species specificity, there are many different CMVs, e.g. human CMV, rhesus CMV (rhCMV) or murine CMV (MCMV) (reviewed in [1]).

CMVs have very large genomes, e.g. the HCMV genome is ~230 kilo bases (kb) long and the MCMV genome is ~235 kb in size [10]. The genome structure differs between CMV species, as some have large inverted repeats, whereas others have small repeat elements [11]. HCMV has a special structure among the *betaherpesvirinae*, because its genome is composed of the U_L and U_S components, which are flanked with direct and inverted repeat elements (reviewed in [3]). Several hundred open reading frames

(ORFs) have been identified in the genome of CMVs, but not all of them have been demonstrated to encode proteins [12, 13].

Analysis of the CMV gene expression kinetic has shown that their genes are expressed in three overlapping phases: immediate-early (*ie*), early and late [14, 15]. Immediate-early genes are the first genes expressed after infection, under the control of strong transcriptional enhancers which can be activated by cellular transcription factors (reviewed in [16]). The immediate-early proteins interact with cellular anti-viral proteins and in the initial phase of infection they are required to activate the expression of early and late genes [17-20]. The translation of the *ie* genes can be suppressed by the protein synthesis inhibitor cycloheximid (CHX), which in turn blocks the transcription of all other genes, whose expression depends on immediate-early proteins [21]. Early genes are involved in DNA replication, immune evasion and viral progression. Late genes encode the structural proteins which are necessary to assemble the virions (reviewed in [3]). The defining difference between the early and the true late genes is the effect of the DNA-polymerase inhibitor phosphonoacetic acid (PAA) on their expression. The expression of true late genes depends on DNA replication. Thus, in the presence of PAA, only immediate-early and early genes are expressed [21, 22].
In the beginning of CMV research gene functions were studied by the use of spontaneously mutated viruses or upon chemically or physically induced mutations [23, 24]. The introduction of the CMV genome into a bacterial artificial chromosome (BAC) vector simplified the analysis of the function of individual genes by targeted mutagenesis. BAC based mutagenesis allows to maintain the CMV genome in *Escherichia coli* (*E. coli*) and to modify it by using antibiotic resistance as selection marker. This enables the production of attenuated mutant virus, because the wild type virus will not outgrow the mutant virus. Nowadays, various CMVs are available as BACs, e.g. HCMV and MCMV [25, 26].

1.3 Clinical Relevance

HCMV is ubiquitous in the human world population. The seroprevalence ranges from 20% up to nearly 100%. The prevalence depends on age and socio-economic factors, e.g. in adults in less developed countries the seroprevalence is higher in comparison to developed countries (reviewed in [27]). Other factors that influence the probability of HCMV infection are the availability of healthcare services and the hygiene status [28, 29]. Most people undergo primary infection as infants and the risk of infection is increased if the care of children takes place in groups. HCMV can be shed, among

others, via the urine and saliva, which is particularly frequent during the primary infection and a reason for the high contagion risk, particularly between small children [30, 31].

In most cases, the primary infection remains undetected because it is asymptomatic or manifests only mild and nonspecific symptoms, e.g. low-grade fever, fatigue or mild hepatitis. The course of the disease is mild in immunocompetent people, probably because the virus is controlled by the immune system. During pregnancy, primary infection can have serious consequences for the foetus. A congenital infection is caused by the placental transmission of the virus from the mother to the unborn child. For approximately 10% of these children, this can have devastating consequences, ranging from hearing impairment to brain damage, causing mental retardation (reviewed in [32] and [33]).

All groups that have undergone a primary infection, including the immunocompetent individuals, carry the virus for life. As already described in chapter 1.1, herpesviruses establish a life-long persisting infection. During the latent phase of infection, viral particles cannot be detected in the host, but the silenced viral genome is episomally maintained in the nucleus of latently infected cells [34]. Reactivation events occur from time to time, induced for example by stress-induced activation of viral promoters (reviewed in [16]). In immunocompetent hosts these small reactivation centers are controlled by the immune system and prevent the virus from spreading. People who have a compromised immune system, whether by disease or as a result of medication, have significantly more severe symptoms, for example hepatitis, retinitis or enteritis (reviewed in [32]). This affects allograft recipients, patients with the acquired immune deficiency syndrome (AIDS), but also cancer patients receiving chemotherapy. The HCMV-related complications in immunodeficient patients, caused by primary infection or reactivation of latent virus, require the development of efficient treatment options.

Nowadays, common therapies against HCMV include the administration of anti-viral drugs, such as ganciclovir, cidofovir, fomivirsen or acyclovir, although all of these compounds have limitations. The HCMV genome is prone to mutations, which leads to the selection of mutants that are resistant to the anti-viral compounds. Moreover, some medications have serious side effects. For instance, cidofovir cannot be used in patients with renal transplants, because of its nephrotoxicity. (reviewed in [35])

Passive immunization was shown to reduce the risk of congenital CMV disease [36], but a vaccine against HCMV could not be developed until today, probably due to its immune-evasive properties.

1.4 Murine CMV as a Model for Human CMV

The most common and well-established animal model to study CMV infection is the mouse model. MCMV and HCMV coevolved with their respective host species and are uniquely adapted to it. Therefore, the CMVs infecting mice or humans are not identical, but nevertheless evolved genes with related functions.

MCMV and HCMV share similarities in the sequence for several essential ORFs. For example, the proteins M80 and M54 of MCMV, an assembly protein and a DNA polymerase, have sequence and functional homologs in HCMV [37, 38], arguing for a common ancestry of these genes and of the CMVs in general. Other genes have distinct sequences, but similar functions, arguing for converging evolution due to similar selection pressures. Therefore, the overall interaction of MCMV and HCMV with their respective hosts and their strategies to escape the immune system are conserved.

Depending on the infectious dose and on the susceptibility of the mouse strain, MCMV causes similar clinical manifestations as HCMV in humans. In immunocompetent mice the primary infection is almost asymptomatic, but the suppression of the immune system of latently infected mice leads to severe organ disease, e.g. pneumonia or retinitis [39, 40]. In contrast to HCMV, MCMV is not placentary transmitted, but newborn mice infected with MCMV develop diseases similar to the infected human newborns, including the infection of the central nervous system [41, 42].

In studies with AIDS patients or organ recipients it could be determined that the $CD4^+$ and $CD8^+$ T cell responses play a critical role in primary HCMV infection or recurrence [43, 44]. The same phenomena were observed in the mouse model [45, 46]. Moreover, the cellular and molecular mechanism of the interaction between virus and the immune system could be studied in minute detail by experiments with knock-out mice, adoptive transfers or monoclonal antibody-mediated depletions of individual components of the immune system.

In conclusion, while the mouse model has limitations to investigate the interaction of HCMV and its host, it has significantly contributed to the understanding of the biology of CMV infections. Therefore, the findings from the mouse model may help to develop new drugs and therapies against HCMV.

1.5 MCMV Latency and Reactivation

MCMV and all members of the *Herpesviridae* family are able to establish a life-long infection in the host. Characteristics of such an infection are an acute phase of viral replication and spread in the host, followed by a latent phase in which the silenced viral genome is detectable in host cells, but only sporadic replication occurs (reviewed in [47]).

Whether CMVs establish a true latency or if infectious virus persistently replicates below the detection threshold is still unclear. Latency is defined as the long-term maintenance of viral genomes in the absence of infectious viral particles and the ability of the virus to reactivate from the latent state. In contrast, a persistent infection is characterized by the permanent presence of replicating viral particles on a low-level. At the organism level, it is difficult to exclude if CMV is persistently replicating in some parts of the body. For example, infectious viral particles are frequently absent from the lungs of an MCMV-infected mouse despite the presence of replicating virus in the salivary glands of the same animal [48].

It is unlikely that CMV establishes latency in one single cell type, because MCMV genomes could be identified in various organs in latently infected mice, for example in the bone marrow, spleen, lungs, liver, kidney and in the heart [49-52]. Seckert *et al.* have shown that the liver sinusoidal endothelial cells (LSECs) are a site of MCMV latency in the liver [53]. Others have shown that latent viral genomes may be found in peritoneal Macrophages [54, 55]. However, it remains unclear, whether macrophages are really a site of latency, since viral genomes have been detected in bone marrow resident precursor cells of the myeloid lineage [55], and hence of monocytes and eventually macrophages. Bone marrow cells and their derivates, the Monocytes, have been also detected as a site of HCMV latency [56-59], and their differentiation into macrophages was shown to result in virus reactivation [60]. The HCMV latency in endothelial cells remains controversial, since viral genomes were detected in endothelial cells of the human aorta [61], although it could not be identified in the saphenous vein [62].

The molecular mechanisms allowing that latency is established and maintained remain unclear. There are two competing models of latency establishment. Both models assume that an infected cell which has once begun to produce viruses cannot become a latently infected cell, because it inevitably dies by apoptosis or lysis. However, one model is based on the idea that viral proteins promote latency together with cellular repressors, which prevent promoter activity [63]. The alternative model assumes that

the genomes are silenced immediately upon infection (reviewed in [64]). Therefore, according to this model, the decision of a lytic or latent infection might already be made after the release of the viral DNA into the cell nucleus.
The infected cells recognise viral proteins or viral DNA by receptors sensing pathogen associated molecular patterns and reacts to viral infection by expressing anti-viral compounds [65].

The initial viral gene expression occurs from the major immediate-early enhancer promoter (MIEP) [66] (reviewed in [3]). The MIEP is bidirectional in the MCMV genome, and drives the expression of the immediate-early genes *ie1*/*ie3* and *ie2* [67]. The genes *ie1* and *ie3* are expressed from the same promoter and share the first three exons, while alternative splicing of exon 4 or 5 results in transcription of *ie1* or *ie3* messenger RNA (mRNA), respectively [17]. The protein IE1 was shown to be dispensable for viral growth *in vitro* and for the establishment of latency, as well as for reactivation. However, the viral growth of an MCMV lacking *ie1* is somewhat attenuated *in vivo* [68, 69]. This may be caused by the fact that IE1 promotes viral gene transcription by dispersing Nuclear Domain 10 (ND10) bodies, which are intranuclear defence mechanisms to inhibit viral transcription [20]. In contrast, the protein IE3 is essential for viral growth *in vitro* and in *vivo* [18]. It is critically important for the expression of essential early genes, because it counteracts cellular defence mechanism and recruits viral and cellular proteins to viral DNA replication domains [19]. The protein IE2 is expressed in the opposite orientation relative to IE1 and IE3 and is completely dispensable for viral growth *in vitro* and *in vivo* and its function is still unknown [70]. During the lytic infection, expression from the MIEP occurs in both directions, resulting in the transcripts of *ie1*, *ie3* and *ie2* mRNAs [71, 72]. New data from my research group indicate that the direction of the expression from the MIEP might depend on the cell type [73].

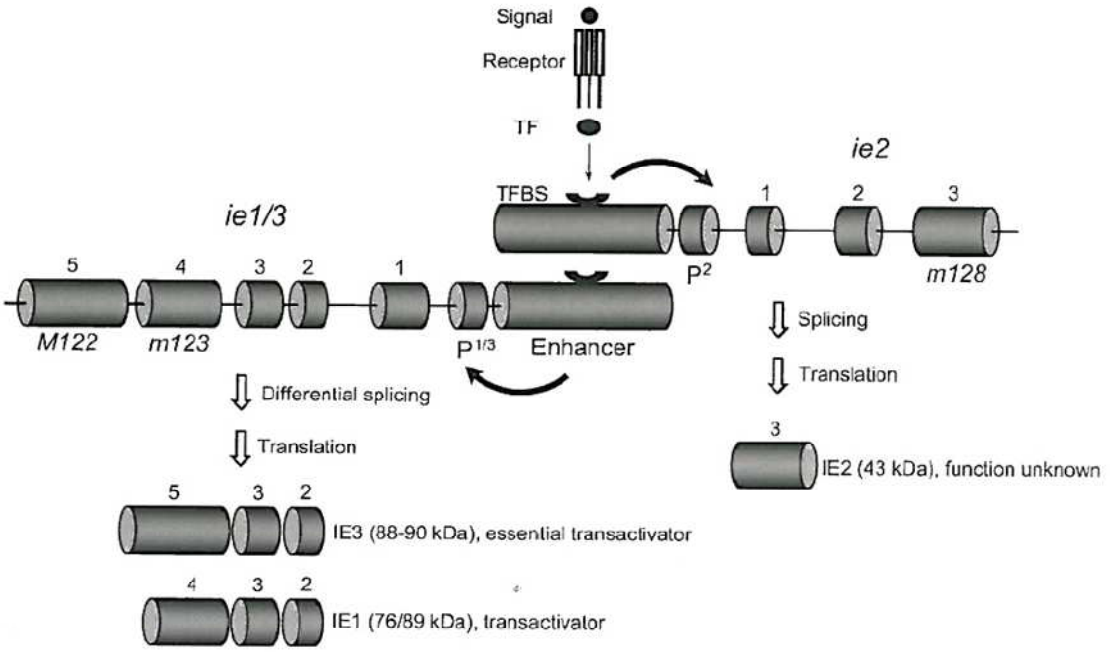

Figure 2 Structure and organisation of the MIE region of MCMV.

The entire major immediate-early enhancer (MIE) region including the exons and introns of the genes *ie1/3* and *ie2* and their related proteins is shown. TF: Transcription factor, TFBS: Transcription factor binding site, P: Promoter. (from Simon et al., 2006, [47])

After the viral genome has entered the cell nucleus, cellular repressors and histones are recruited to the incoming, foreign DNA [74]. Histones can be associated with the repression of promoter activity, by being deacetylated or methylated. It has been shown *in vivo* that in latently infected cells the cellular repressors YY1, CBF1/RBP-Jk, CIR and Daxx are bound to the MIEP [74]. YY1 is a transcription factor, which can act as an activator or repressor. In HCMV infection, YY1 was shown to repress the *ie1* gene expression [75]. CBF1/RBP-Jk is a protein which recruits histone deacetylases (HDACs) and other corepressors to its binding site (reviewed in [76]). CIR is a corepressor, which is recruited to the MIEP to inhibit viral gene expression [74] and Daxx is a compound of the anti-viral ND10 complex (reviewed in [77]). The histones associated with the MIEP during latency are hypoacetylated and hypermethylated, whereby the MIEP is mostly transcriptionally inactive [78].

Since CMV lytically replicates in various cell types, the virus needs to overcome anti-viral responses by different host cells. The viral proteins IE1 and IE3 are not only transactivators of early and late viral gene expression, but are also counteracting the intrinsic cellular anti-viral response [18-20]. Abundant expression of these proteins disperses, the cellular repressors, allowing the RNA polymerase II to bind to the viral promoters and initiate transcription of viral genes (reviewed in [64]), [74].

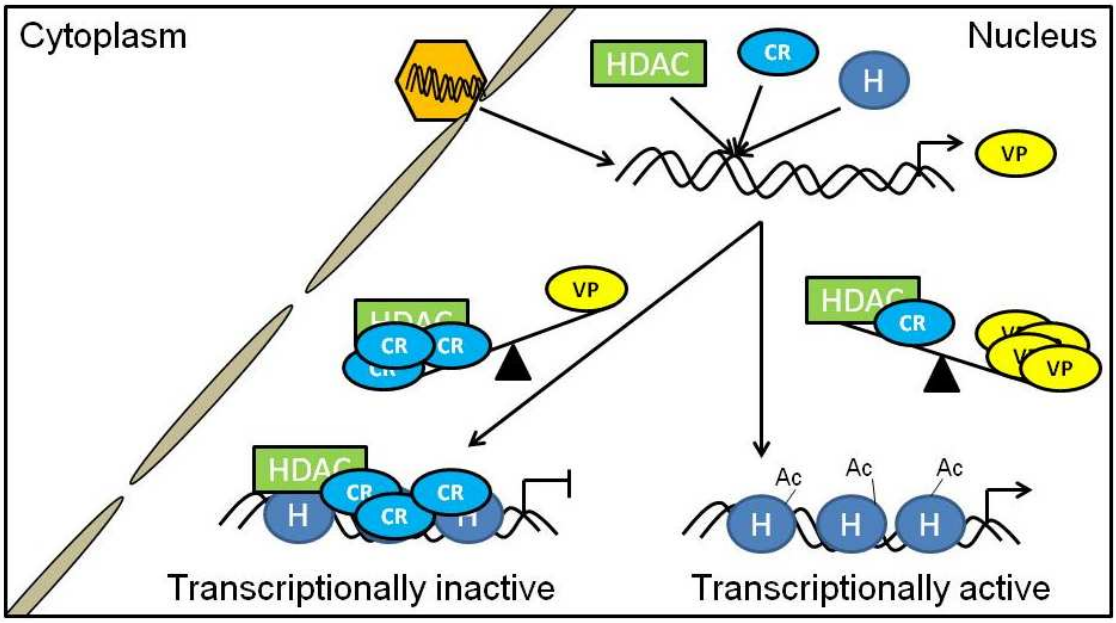

Figure 3 Repression and activation of viral gene transcription.

Upon entering the cell nucleus, the viral genome is packed with histones, and cellular repressors bind to viral promoters. The overexpression of viral proteins can reverse the transcriptional repression, which leads to transcriptionally active viral promoters. HDAC: Histone deacetylase, CR: cellular repressors, H: Histone, VP: viral proteins, Ac: acetylated histone.

These data have led to the hypothesis that the virus can establish latency if the cellular repressors silence the MIEP successfully before the immediate-early genes *ie1* and *ie3* are overexpressed (reviewed in [64]). If this does not occur, the expression of viral IE proteins counteracts the cellular repressors, allowing the virus to enter the lytic cycle. Both scenarios have advantages and disadvantages for the virus. The lytic infection of cells allows the virus to spread in the body, but the latent infection is required for viral long-term maintenance in the host.

The adaptive immune system also contributes to the repression of viral replication. Although MCMV has developed strategies to escape recognition by immune cells (reviewed in [79]), it cannot completely prevent that the infected cell presents viral antigen on its surface and thus can be recognised by cells of the immune system, especially if the virus tries to reactivate from latency [80].

However, the transcription of viral genes during latency is not completely suppressed, since the transcription from the MIEP occurs sporadically. mRNA of the genes *ie1* and *ie2* could be detected in the lungs of latently infected mice while transcripts of the

genes *ie1* and *ie3* were identified in latently infected LSECs [53, 71]. This occurred in the absence of early and late genes transcription, which argues against a reactivation of the virus [81].

Continuous modification of histones takes place in metabolically active cells, which might lead to a temporary opening of the MIEP, allowing the binding of RNA polymerases. Hence it is assumed that the virus reactivates after a change of the cell metabolism or of the cell environment. This notion is supported by MCMV reactivation from latently infected macrophages upon stimulation with thioglycollate [54]. Furthermore, the MIEP has binding sites for inflammation or stress induced transcription factors, like NFκB and AP-1 (reviewed in [16]). Therefore a pro-inflammatory environment may promote viral reactivation [82]. Virus reactivation may also be induced by the elimination of the adaptive immune system, such as $CD4^+$ T cells, $CD8^+$ T cells, B cells and natural killer cells (NK cells) [83]. It remains unclear whether the reactivation is due to the lack of the interaction of immune cells with the latently infected cell or whether this may occur due to a change of the cytokine profile associated with the depletion. Nevertheless, both the increased production of cytokines and the absence of the immune system may be triggers for viral reactivation after organ transplantation. Interestingly, experimental allogeneic kidney transplants induced an increase of inflammatory cytokines in the transplanted tissue and MCMV reactivation of latency [84].

In summary, the reactivation of latent CMV can be triggered by several factors. These factors include cellular differentiation and enhanced cellular activity accompanied with the remodelling of the chromatin structure, the down-regulation of repressive transcription factors and up-regulation of activating transcription factors. In addition, the production of inflammatory cytokines upon tissue damage can induce CMV reactivation [84]. One might argue that the capacity of CMV to reactivate from latency is a survival strategy, developed over millennia, to escape from injured or moribund hosts.

1.6 The Role of the Innate Immune System in MCMV Infection

The innate immune system is the first line of defence against an infection. Components of the innate immune system include the complement system, cytokines, NK cells and professional antigen-presenting cells (APCs) like macrophages and dendritic cells (DCs).

The complement system consists of different proteins, which bind to opsonised pathogens or to the pathogen's cell surface and induce its lysis. Thus, it is involved in the clearance of infections.

The activation of the immune system is mediated by cytokines and phagocytic cells. Professional APCs internalise pathogens and apoptotic bodies from infected cells, processing the degraded proteins and presenting them to cells of the adaptive immune system. Moreover they recruit other immune cells to the site of infection by secreting cytokines.

Interferons (IFNs) are cytokines which play an important role in the control of viral infection. Relative to the receptor they bind to, there are two main classes of IFNs: type I IFNs (IFN-I) and type II IFNs (IFN-II). The IFN-I binds to IFN-I receptors and consists of IFNβ and IFNα variants, whereas there is only one IFN binding to IFN-II receptor: IFNγ. IFN signalling leads to the nuclear translocation of STAT molecules and expression of multiple anti-viral genes. IFNγ signalling is dependent on the phosphorylation of STAT1 homodimers, whereas IFN-I signalling needs STAT1 and STAT2 heterodimers. Due to the different STAT molecules involved in downstream signalling, the activation of IFN-I or II receptors results in the expression of different genes. (reviewed in [85] and [86])

The expression of IFNγ and its signalling via the IFN-II receptor decreases viral growth (reviewed in [85]) [87] and promotes the expression of major histocompatibility complex (MHC) class II (MHC-II) in macrophages [88] and MHC class I (MHC-I) in all cells (reviewed in [89]). Splenic stromal cells in the marginal zone produce high amounts of IFN-I in response to MCMV infection, which activates immune cells like NK cells and recruits them to the site of infection [90]. DCs are responsible for the second wave of IFN secretion in MCMV infection [87]. They are classified into two groups, based on their surface markers and function: plasmacytoid DCs (pDCs) and conventional DCs (cDCs). The pDCs localise mainly to the blood and the lymphoid tissues. Upon detection of an infection, they release large amounts of IFNα and present antigens on their cell surface to activate other immune cells, e.g. NK cells [91]. The cDCs are found in lymphoid and non-lymphoid tissues and are divided in many different subsets. (reviewed in [92]). cDCs which express the co-receptor CD8 have an important role in

priming $CD8^+$ T cells via cross-presentation during acute MCMV infection [93] (reviewed in [94]). DCs are also important early upon MCMV infection because they activate NK cells and promote the expansion of $Ly49H^+$ NK cells [95].

NK cells are involved in the controlling of tumour cells and viral infections, by the production of cytokines and cytotoxic activity. They express activating and inhibitory receptors simultaneously to distinguish between healthy and degenerated or virus-infected cells (reviewed in [96]). The inhibitory receptors recognise MHC-I molecules and prevent the cytotoxic activity of the NK cell. Virus-infected cells often down-regulate the presentation of MHC-I molecules to evade the cytotoxic T lymphocyte (CTL)-mediated killing (reviewed in [96]). In the absence of MHC-I the binding of activating receptors of NK cells to their ligands prevails (e.g. NKG2D binding to Rae-I [97], Mult-1 [98, 99] or H60 [100, 101]) which initiates the killing of the infected cell (reviewed in [102]).

NK cells may also become highly activated through the binding of the Ly49H receptor to its ligand, the MCMV protein m157 [103, 104]. The expression of the Ly49H receptor is strain dependent; C57BL/6 mice express it, and hence their NK cells provide strong immune protection against MCMV infection. In contrast, Balb/c mice and other Ly49H-deficient strains, are susceptible to MCMV infection [105]. NK cells act against the virus-infected cells by secreting cytotoxic granules, IFNγ and by helping in activating the T cells [106, 107]. The NK cell role in the control of CMV is evolutionarily conserved and they are not only important to combat MCMV [108-110], but also HCMV [111].

1.7 The Role of the Adaptive Immune System in MCMV Infection

The cells of the adaptive immune system are divided into B and T lymphocytes. The B cells mediate the humoral response to infections, while the T cells are responsible for the cellular response. The humoral response is also called the antibody-mediated response, where antibodies target the pathogens in the extracellular body fluids. In the cellular response, the T cells target the infected cells and thus the intracellular pathogens. Both subsets play an important role during the acute infection with MCMV, but also during its reactivation. While the T cells are able to control a CMV infection without B cells [83], the B cells alone are not able to combat CMV [112].

1.7.1 The Humoral Response to Infections

B cells are activated by recognising viral antigens with their immunoglobulin (Ig) receptors on the cell surface. In the case of protein antigens a signal cascade leads to the internalisation of the antigenic protein bound to the B cell receptor (BCR), followed by processing and presentation of protein-derived peptides on MHC-II. B cells require the help of the peptide-specific $CD4^+$ T-helper cells to become fully activated. Upon $CD4^+$ T cells stimulation, the B cells proliferate and form germinal centres in secondary lymphoid organs, which leads to B cell differentiation into plasma cells and establishment of B cell memory (reviewed in [113, 114]). Interaction between the follicular $CD4^+$ T-helper cells and B cells initiates the Ig class-switch of B cells, resulting in the secretion of antigen-specific IgG antibodies. The binding of antibodies to viral antigens can neutralise the viruses and thus inhibit the viral *in vivo* dissemination. Furthermore, opsonisation of viruses by antibodies labels them for detection of components of the innate immune system e.g. the complement system or the phagocytic cells. MCMV and HCMV may be neutralised by antibodies against the viral surface glycoprotein B (gB) [115, 116].

The role of antibody-secreting B cells in MCMV infection was defined in experiments with B cell-deficient mice. The presence of MCMV-specific antibodies does not significantly decrease viral titres in organs during acute infection, but limits the spread of the virus upon reactivation caused by immune suppression of latently infected mice [83]. The establishment of latency, however, is not prevented [117, 118]. In clinical tries, the passive immunisation is experimentally used as therapy, to suppress the symptoms of HCMV infection in immunocompromised patients or foetuses [36].

1.7.2 The Cellular Response to Infections

The cellular response to infections is mediated by T cells. They can either directly act on infected cells inducing their apoptosis, or facilitate the activation of phagocytic cells which subsequently engulf apoptotic bodies or pathogens.

Lymphoid cells of the B cell lineage undergo their maturation in the bone marrow, but the maturation of T cells occurs in the thymus. The T cell maturation in the thymus is divided into several steps, starting with immature T cells expressing neither CD4 nor CD8, transitioning through the double-positive ($CD4^+$ $CD8^+$) phase and ending with naive T cells which express either the CD4 or the CD8 co-receptor (reviewed in [119]). $CD4^+$ T cells are termed T-helper cells, because they activate immune cells and promote the survival of other T cells (reviewed in [120]). The co-receptor CD4 binds to

the constant part of the MHC-II, which is mainly expressed on APCs. The co-receptor CD8, expressed on CTLs, facilitates the binding to the MHC-I (reviewed in [121]).

The binding to the MHCs is facilitated by the T cell receptors (TCRs), expressed on every T cell. Due to a selection process during the T cell maturation, each T cell clone expresses a different TCR, which is composed of a constant region and a variable region, by which it binds to the MHC. The somatic recombination of the variable (V), the diversity (D) and the joining (J) gene segment during the maturation process rearrange the VDJ locus into a sequence that differs from each cell (reviewed in [122]).

The process depends on the recombination-activating genes 1 and 2 (RAG-1 and RAG-2) thus ensuring that each T cell gets a unique TCR with different combinations of the V, D and J segments, but also of additional codons introduced at random during the rearrangement process (reviewed in [123]).

The MHC-I is expressed on every nucleated cell within the body. MHC-I present constantly short peptides on the cell surface, which are derived from the proteins that the cell has produced. Old or defective proteins are degraded by the proteasome and the resulting peptides are transported via a transporter complex, called transporter associated with antigen processing (TAP), into the endoplasmatic reticulum (ER). The peptide loading complex, that allows the loading of the peptide on the MHC-I, is directly bound to the TAP. Peptides are actively transported via the TAP and loaded onto the MHC-I. The peptide loaded MHC-I then may dissociate from the loading complex and is transported via the Golgi to the cell surface, where cells of the immune system can bind to and initiate cell death once an antigenic peptide (e.g. from a virus) has been recognised. (reviewed in [124] and [125])

Efficient binding of the TCR to the MHC-I, depends on the peptide presented by the MHC and the presence of co-stimulatory molecules on the surface of the target cell. The expression of co-stimulatory ligands is stress-induced, for example in an infected or degenerated cell. These ligands increase the signal transduction and prolong the binding of the T cell to its target, contributing to the activation of T cells. The T cell responds with the secretion of molecules resulting in the activation of the apoptotic pathway in the target cell, if a strong signal is provided by multiple TCRs bound to their ligands. (reviewed in [126] and [127])

However, some pathogens have developed strategies to escape this control mechanism. MCMV acquired several genes counteracting the peptide presentation on MHC-I, termed immunoevasions (Figure 4). For example, m152 encodes for a protein which retains the MHC-I in the ER and prevents its transport to the Golgi [128, 129].

Another protein m06 leads to the transport of MHC-I from the ER into a lysosome and to its degradation [130]. HCMV acquired proteins with similar functions [131-133].

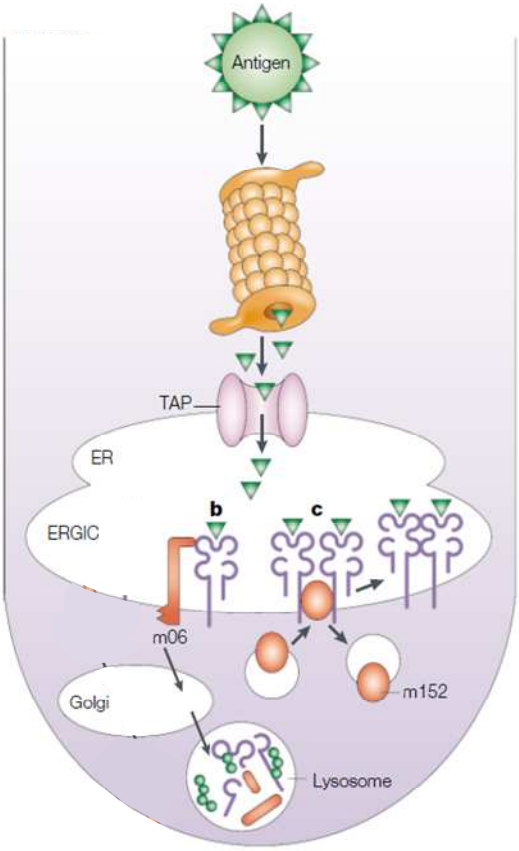

Figure 4 Model for the action of two immunoevasions of MCMV.

The antigen is degraded by the proteasome and peptides are transported via TAP into the ER, where the peptides are loaded onto the MHC-I. The Protein m152 retains the MHC-I in the ER and prevents its presentation on the cell surface. The protein m06 leads the MHC-I to be transported to the lysosomes and thus, to its degradation. TAP: transporter associated with antigen processing, ER: endoplasmatic reticulum, ERGIC: ER-Golgi intermediate compartment. (modified from Reddehase, 2002 [134])

The activation of T cells usually takes place in lymphoid organs. In response to an infection, professional APCs, in particular DCs mature and migrate to the lymphoid organs, such as spleen and lymph nodes. Here they encounter the resting naive and memory T cells, present them the antigenic peptides, resulting in T cell activation (reviewed in [135]). APCs engulf and digest dead cells in a site of inflammation and the short peptides derived from the degraded proteins are loaded on the MHC-I and MHC-II and presented to $CD8^+$ and $CD4^+$ T cells, respectively. The activation of $CD8^+$ T cells may also occur if the APC is infected with intracellular pathogens such as viruses and the antigenic peptides are presented directly on the MHC-I of the infected cell.

Moreover, naive $CD8^+$ T cells need additional cytokine signals for activation. The proliferation of a T cell specific for the peptide presented on the MHC results in a population of T cell clones with the same TCR capable of targeting and neutralising antigens. (reviewed in [136])

Most of the expanding T cells are specific for only a few antigenic peptides, termed immunodominant peptides. Besides other characteristics, these peptides are preferentially generated and loaded on the MHC and able to trigger the most intense immune response, (reviewed in [137]). Upon antigen encounter, T cells expand and differentiate into effector or memory cells, depending on the presence of the corresponding inflammatory cytokines. The activated effector T cells down-regulate receptors responsible for their residence in the lymphoid organ and up-regulate receptors specific for binding to endothelial cells in blood vessels. Thus, they leave the central lymphoid organ, enter the peripheral circulation and migrate to the site of infection, where they are able to leave the blood vessels by active extravasation (reviewed in [138]). Some T cells differentiate into memory T cells upon antigen encounter and reside in organs and lymphoid organs for the rest of their life (reviewed in [139]). The clonal expansion and maturation of effector T cells requires time to provide sufficient amounts of fully activated cells. Therefore, the innate immune system keeps the pathogens in check immediately upon infection, until more adaptive immune cells are recruited and expanded. If the infection is successfully controlled, the cytokines that promote T cell proliferation are down-regulated and thus the T cells lack the signals that they need for survival. As a result, they undergo apoptosis [140]. However, the memory T cells may survive for a long time in the lymphoid organs, because here they are exposed to additional survival stimuli, for instance interleukin(IL)-15 (reviewed in [141]).

In an ongoing infection, three subsets of $CD8^+$ T cells emerge: naive T cells, central memory T cells (T_{CM}) and effector memory T cells (T_{EM}) (reviewed in [142]). These subsets can be distinguished by the expression of the receptors CD44, CD62L or CD127 [143]. Naive T cells have low expression of CD44, but high expression of CD62L and CD127. CD62L, known as L-Selectin, is an adhesion molecule which binds to a ligand mainly expressed on cells of the secondary lymphoid organs [144]. CD127 is the alpha chain of the receptor for IL-7, a cytokine which is important for the survival of naive and memory T cells (reviewed in [145]). T_{CM} cells are characterised by the high expression of CD62L, CD127 and CD44. CD44 is an adhesion molecule which is expressed on activated T cells and promotes the extravasation of T cells to sites of

inflammation [146]. Effector and effector memory T cells express low amounts of CD62L, show low or intermediate expression of CD127 and high expression of CD44 [143].

During an infection, primed naive T cells undergo the expansion and the differentiation phase, resulting in large amounts of fully functional effector T cells. When the acute infection is cleared, up to 95% of the pathogen-specific effector T cells die, while the remaining cells from the memory T cell pool persist in the long-term, even in the absence of antigen (Figure 5). This pool of memory cells is mainly composed of T cells with a T_{CM} phenotype (reviewed in [142]).

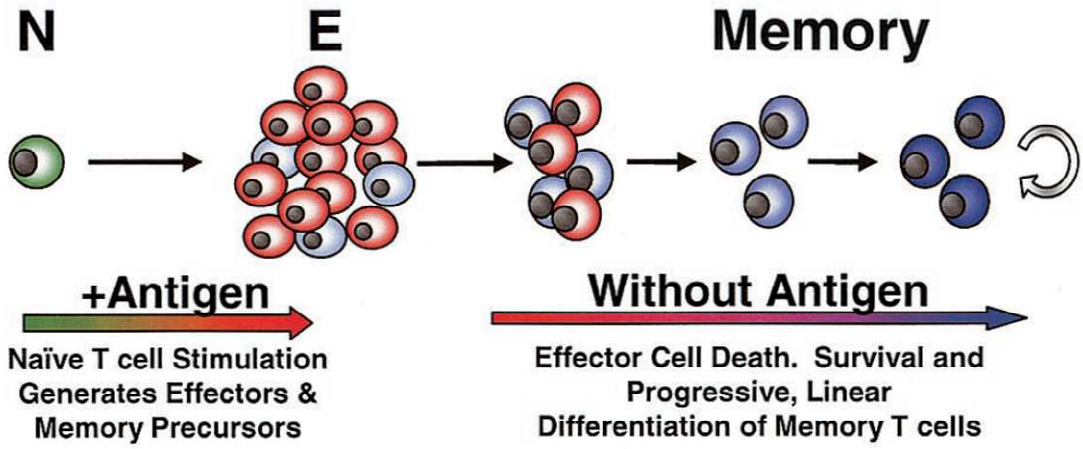

Figure 5 Dynamic of $CD8^+$ T cells during and after an infection.

Naive T cells are stimulated by an antigen and proliferate and differentiate into effector T cells and memory T cells. Without the stimulating antigen, e.g. after the infection is cleared, effector T cells undergo cell-death, while the memory T cells remain in the long term. N: Naive T cell; E: Effector T cell. (from Wherry, E.J. and Ahmed, R., 2004 [143])

1.7.3 Modulation of the Cellular Immune System by CMV

During CMV infection, some of the virus-specific T cells undergo the typical fast expansion in the acute phase, followed by a contraction after the infection is cleared and are marked by T_{CM} phenotype [147] (Figure 6). On the other hand, several CMV-specific T cells accumulate (or inflate) over time and do not contract, a phenomenon termed memory inflation (MI) [148]. These T cells are specific for the certain peptides and have a T_{EM} phenotype, characterised by low expression of CD62L and CD127 receptors on the cell surface [147, 149, 150].

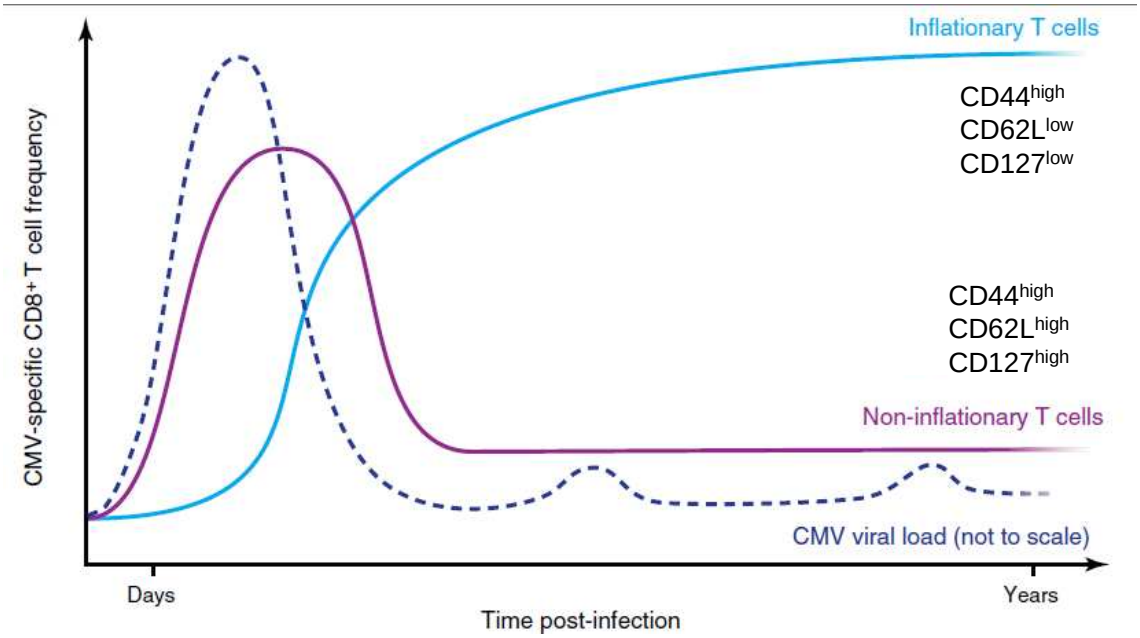

Figure 6 Development of CMV-specific memory CD8$^+$ T cells.

Non-inflationary T cells expand in the acute phase of infection, contract after the virus infection is cleared and the memory T cells remain on a low level. These cells develop a T_{CM} phenotype. Inflationary T cells expand slowly in the acute phase of infection but continue to accumulate during latency. These cells develop a T_{EM} phenotype. (modified from O'Hara et al., 2011 [151])

In latently infected people, ~10% of the CD4$^+$ and CD8$^+$ T cells are HCMV-specific, with the consequence that these T cells dominate the T cell pool [152]. Similar results were observed in mice, where one year after the infection already 20% of the CD8$^+$ T cells were found to be specific for one single MCMV-epitope [148]. During the acute phase of infection, a broad T cell response is observed against different MCMV epitopes. In the latent phase, however, five inflationary viral epitopes dominate the pool of MCMV-specific T cells in C57BL/6 mice [147]. This strong response affects the T cell pool in general, because the absolute count of CD8$^+$ T cells is significantly higher in MCMV-infected mice than in uninfected ones [153].

Why only some CMV-specific T cells show this accumulation is not fully understood, but several contributing factors have already been identified. It has been shown that the accumulation of inflationary T cells is not impaired in mice which are unable to perform cross-presentation [93]. However, the presence of CD4$^+$ T cells promotes MI [46, 154]. Furthermore, it was recently shown that the context of gene expression and competition for antigen presentation plays a role in the dominance of individual peptides during latency [155, 156]. The expression pattern of the gene which encodes the peptide influences the kinetic of the peptide-specific T cells. A foreign peptide inserted in an immediate-early gene results in inflationary T cells, whereas the same peptide inserted in an early gene results in non-inflationary T cells. Others have shown

that the initial viral replication, or continuous replication events during MCMV latency is not necessary to drive MI. Systemic infection with a spread-deficient MCMV, which lacks the essential glycoprotein gL and is therefore unable to replicate, drove the accumulation and maintenance of MCMV-specific $CD8^+$ T cells with a T_{EM} phenotype [157]. It is therefore assumed that persistent viral gene expression triggers MI. It remains unclear, how the pool of virus-specific T cells sustains its number over time. While the recruitment of novel naive T cells into the pool of inflated cells is in theory possible, MI occurred to the same extent in thymectomised mice [158]. It was shown that CMV-specific T_{EM} cells are short-lived, because their numbers decrease after being adoptively transferred into a recipient mouse, infected with MCMV, and it is therefore unlikely that they have proliferative potential [150]. Hence, it is assumed that the size of the T_{EM} cell pool is maintained by the recruitment of other T cells. On the other hand direct antigen presentation by non-hematopoietic cells was shown to drive the proliferation of MCMV-specific T cells in the lymph nodes during latency [159]. There, it was shown that adoptively transferred T_{CM} cells from latently infected secondary lymphoid organs can proliferate in recipient mice that are also MCMV-infected, in contrast to T_{EM} cells from latently infected lungs, which failed to expand upon adoptive transfer.

Since T_{CM} cells are more abundant than T_{EM} in the lymph nodes, it is considered that local persistent viral gene expression stimulates the T_{CM} cells to proliferate, differentiate into T_{EM} cells and to migrate out of the central lymphoid organs into the periphery and the organs. On the other hand, a proof for the continuous proliferation and replenishment of the T_{EM} cell pool by T_{CM} progenitors rests on experiments with adoptively transferred cells, but a direct proof in absence of adoptive transfer has remained elusive.

1.8 Conditional Gene Expression System (Cre/*loxP*)

One method to investigate the function of a protein in an organism or a pathogen is to silence its gene, e.g. by the removal of the coding gene sequence from the genome, or by the introduction of a nonsense mutation. The function of the missing protein may be thus identified by the change in phenotype. However, if the silenced gene is essential, it will inevitably lead to the death of the organism and therefore the study of the exact function of this protein will be severely limited. In such cases it is beneficial if the expression or the function of the protein can be controlled in an inducible manner. This can be achieved, for example, with temperature-sensitive mutations, in which an

increase or decrease of the temperature results in a non-functional protein, although this exact method cannot be used for organisms which have to keep a constant body temperature (e.g. mammals or birds).

The Cre/*loxP* system is a genetic system that offers an opportunity to conditionally control the gene expression. The enzyme Cre and its target, the *loxP* sequence, are derived from the bacteriophage P1 [160]. Cre means literally “causes recombination” and is a 38 kDa enzyme which acts *in trans* to promote recombination. The sequence on which the enzyme acts on is called *loxP* site: locus of crossing over (x), P1. If two *loxP* sites, 34 base pairs (bp) in length, flank a gene locus, Cre recombinase may recombine the intervening sequence. If two directly repeated *loxP* sites flank the gene, the sequence between them is excised, whereas DNA segment flanked by inverted *loxP* sites are inverted in the presence of Cre recombinase [161, 162]. The Cre-mediated site-specific recombination at *loxP* sites works in mammalian cell lines, as well as *in vivo* [163]. Mating mice which express the Cre enzyme with a mouse line which has a DNA segment flanked with *loxP* sites leads to the excision of the intervening segment in the murine progeny [164].

Various mouse strains expressing the Cre enzyme under the control of different promoters are readily available, allowing ubiquitous as well as tissue-restricted expression of Cre (reviewed in [165]). It has been shown in several studies that the Cre/*loxP* system also works when their components were integrated into viral genomes including herpesviruses like MCMV [166-169]. However, even if the use of the Cre/*loxP* system has great advantages in the investigation of tissue-specific activity of proteins, the function of genes whose expression is essential for the survival of the organism cannot be studied. This limitation has been mitigated by the development of a system in which the activity of the Cre enzyme can be temporarily controlled.

To produce an inducible Cre enzyme, the ligand-binding domain of the oestrogen-receptor (ER) was fused to the Cre [170]. Upon activation by oestrogen binding, the oestrogen receptor translocates into the nucleus and activates the transcription of oestrogen inducible genes [171]. The activity of Cre was controlled by the addition of an oestrogen, estradiol or its antagonist 4-hydroxytamoxifen. Both bind to the oestrogen-receptor and lead to the translocation of Cre into the cell nucleus. Without estradiol or Tamoxifen (Tam), the Cre-ER remains in the cytoplasm due to the interaction of the oestrogen receptor with the heat-shock protein 90 (Hsp90) [172, 173]. Due to uncontrolled nuclear translocation caused by naturally circulating oestrogen, the ER receptor cannot be used for targeted induction of Cre activity *in vivo*. To circumvent this problem, a mutated murine oestrogen receptor (ER^T) was developed, which cannot

bind oestrogen, but remains sensitive to 4-hydroxytamoxifen [174, 175], with no or only minor background recombination in the absence of Tam [176-178]. A mouse line expressing this fusion-protein Cre-ER^{T} was created, in which the enzyme is expressed under the control of the Rosa26 (R26) promoter [179]. The R26 locus is expressed ubiquitously in embryonic and adult tissues and thus is highly suitable for ubiquitous Cre expression [179, 180]. This system allows the temporal control of Cre activity by the administration of Tam and thus the excision of DNA segments, flanked with *loxP* sites (Figure 7).

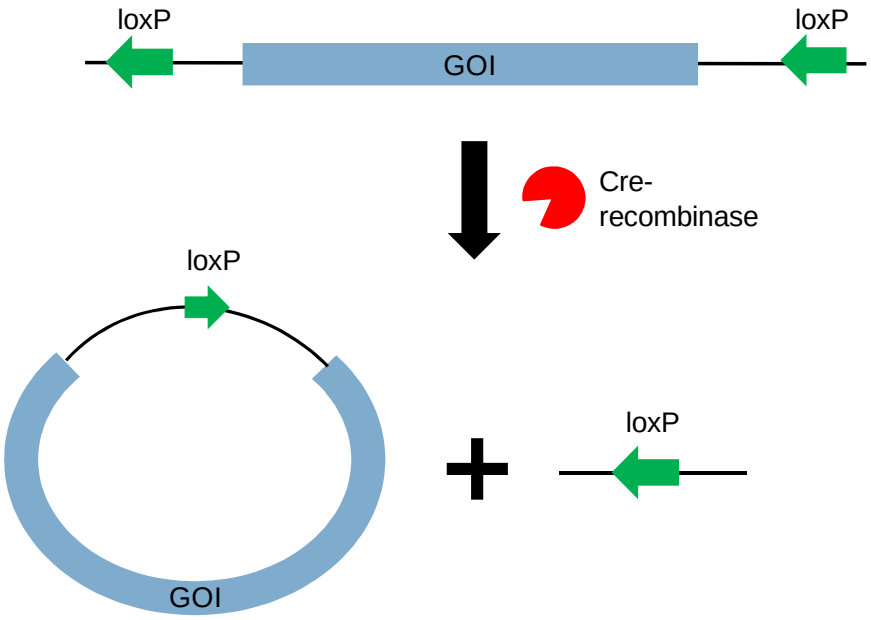

Figure 7 Overview about an induced knock-out by the Cre/*loxP* system.

The ORF of the gene of interest (GOI) is flanked by *loxP* sites. The enzyme *Cre recombinase* binds to the *loxP* sites and recombines the GOI. What remains is the genome without the GOI and a plasmid-like structure.

The most common routes of Tam administration are the administration into the stomach with a feeding needle and the injection into the abdominal cavity [181-183]. However, both methods are applied for a period of 5 days only, because they are associated with high levels of stress for the animals. Alternatively, the administration can be done via food pellets, enriched with a certain concentration of Tam [184] and the concentration of 360 mg/kg is described as sufficient to achieve recombination by Cre, fused to the mutated murine oestrogen receptor ER^{T2} (Cre.ER^{T2}) [184]. The efficiency of recombination of Cre.ER^{T2} is not described yet, when induced by Tam administration via chow and expressed from the Rosa26 promoter. With oral administration or intraperitoneally (i.p.) injection of Tam for 5 days, recombination could

be achieved in all tissues of the adult animal [176], with the exception of the brain, where no Cre.ER^{T2} expression was observed. This circumstance is of minor importance for studying MCMV T cell responses in the blood or secondary lymphoid organs, because MCMV infection of the brain has not been described until now, if the infection was performed i.p. Up to date, a Cre.ER^{T2}-mediated recombination, induced by the administration of Tam with chow, is described only in systems in which the Cre recombinase is expressed under cell type-specific promoters [184-186].

1.9 Objectives of the Work

It is generally accepted that persistent viral gene expression stimulates CMV-specific CD8$^+$ T cells to proliferate. During CMV infection, some virus-specific inflationary CD8$^+$ T cells with a T_{EM} phenotype progressively accumulate through the lifetime of the latently infected host [147, 149, 150]. It is assumed that the large population of MCMV-specific T_{EM} cells is highly dynamic and composed of newly recruited cells, because the short-lived T_{EM} cells are believed to proliferate only partially and therefore contribute only limited to the maintenance of MI [150]. T_{CM} cells, isolated from latently infected secondary lymphoid organs were able to proliferate upon transfer in infected mice, whereas T_{EM} cells isolated from the latently infected lungs did not [159]. However, these results were obtained by adoptive transfers of T cells, thus in an artificial system.

In this study the effect of persistent viral gene expression on the maintenance of MI was to be analysed. For this purpose, the immediate-early genes *ie1* and *ie3* of MCMV were chosen, because their transcription is detectable in latently infected organs months after infection, hence in the absence of viral particles [72]. The *ie1/3* gene locus was to be flanked with *loxP* sequences to generate the recombinant virus MCMV IE1/3^{flox}. The *loxP* sites are targets for the Cre recombinase and leads to the enzymatic excision of the framed DNA fragment. C57BL/6 mice expressing the inducible Cre recombinase Cre.ER^{T2} ubiquitously under the control of the Rosa26 promoter were to be infected with either MCMV IE1/3^{flox} or MCMV wild type (WT). Cre.ER^{T2} is inactive unless it is activated by Tam. The activity of the Cre recombinase could be controlled by administer Tam containing chow to the mice, and switched on once the MCMV infection had reached the latent state. In this phase of infection MI is established and the effect of the *ie1/3* knock-out can be monitored for the further course of CD8$^+$ T cell accumulation.

In the first part of the study, it was found that Tam induced Cre.ER^{T2} had an unexpected direct influence on the composition of the CD8$^+$ T cell pool, mainly by killing the proliferating cells. Due to these results, it was decided to examine the different CD8$^+$ T cell populations in latently infected spleens for their susceptibility to Cre-mediated toxicity. The spleen is a secondary lymphoid organ, in which T_{CM} and naive T cells, as well as T_{EM} cells are located [187, 188], and hence allowed the identification of the T cell subset which underwent apoptosis due to Cre-mediated toxicity. Results might give an indication of the cell type that contributes to the accumulation of inflationary CD8$^+$ T cells.

2 Material and Methods

2.1 Working Concentrations of Antibiotics

Antibiotic	final concentration
Ampicillin (Amp)	50 µg/ml
Chloramphenicol (CAM)	25 µg/ml
Kanamycin (Kn)	20 µg/ml

2.2 Chemicals and Reagents

The chemicals required for the solutions, buffers and media were obtained from the companies ROTH (Karlsruhe, DE), Merck (Darmstadt, DE), Avantor (Center Valley, PA, USA) and Sigma-Aldrich (St. Louis, MO, USA). The antibiotics used were supplied by ROTH (Karlsruhe, DE) and Serva (Heidelberg, DE).

Brefeldin A	cell signalling technology (Danvers, MA, USA)
CompBeads	BD (Franklin Lakes, NJ USA)
Dimethyl sulfoxide (DMSO)	euroclone (Pero, IT)
GelRed	Biotium (San Francisco, CA, USA)
Heparin	Ratiopharm (Ulm, DE)
Isofluran	Abbott (Wiesbaden, DE)
Kanamycin	Sigma-Aldrich (St. Louis, MO, USA)
Millipore water	Merck Millipore (Billerica, MA, USA)
NaPyr (N-nitroso-alpha-acetoxy-pyrrolidine)	life technologies (Carlsbad, CA,USA)
Teklad CRD Tam^{400}/CreER	Harlan Teklad (Indianapolis, IN, USA)
Trypan Blue	Fluka analytic (Sigma-Aldrich)

2.3 Tissue Culture Media and Reagents

Dulbecco's modified Eagle's medium (DMEM)	Sigma-Aldrich (St. Louis, MO, USA)
Fetal Calf Serum (FCS)	biochrom (Berlin, DE)
Glutamine	life technologies (Carlsbad, CA,USA)
IFNß	PBL Interferon Source (Piscataway, NJ, USA)
Fixation buffer	eBioscience (San Diego, CA, USA)
Fugene HD Transfection Reagent	Promega (Madison, WI, USA)
	Liver Digest medium life technologies (Carlsbad, CA,USA)
Liver Digest medium	life technologies (Carlsbad, CA,USA)
Liver perfusion medium	life technologies (Carlsbad, CA,USA)
Opti-MEM I Reduced Serum Medium	life technologies (Carlsbad, CA,USA)
PBS (phosphate buffered saline)	137 mM NaCl
	2.7 mM KCl
	10 mM Na_2HPO_4 * 2 H_2O
	2 mM KH_2PO_4
	adjust pH to 7.4
Penicillin/Streptomycin	life technologies (Carlsbad, CA,USA)
Percoll	biochrom (Berlin, DE)
Permeabilization buffer	eBioscience (San Diego, CA, USA)
Roswell park Memorial Institute medium (RPMI)	Sigma-Aldrich (St. Louis, MO, USA)
Tam for cell culture	Sigma-Aldrich (St. Louis, MO, USA)
Trypsin	life technologies (Carlsbad, CA,USA)

2.4 Buffers and Solutions

Ammonium-Chloride-Potassium (ACK) lysis buffer	155 mM NH_4CL		
	10 mM $KHCO_3$		
	1 mM ETDA		
	adjust with H_2O to 900 ml		
	adjust pH to 7.8 with 1 M NaOH		
	adjust with H_2O to 1000 ml		
	sterilising by autoclaving or sterile filtration		
agarose gel	Biozym (Hessisch Oldendorf, DE)		
D-biotin	0.2 mg/ml		
	sterilising by sterile filtration		
Fluorescence activated cell sorting (FACS) buffer	PBS + 2% FCS		
Gelatine	Sigma-Aldrich (St. Louis, MO, USA)		
Hank's Balanced Salt Solution (HBSS)	AppliChem (Darmstadt, DE)		
L-leucin	10 mg/ml		
	sterilising by sterile filtration		
Luria-Bertani (LB)-full medium	BD (Franklin Lakes, NJ, USA)		
lysis buffer	1 M Tris (pH 8)	2.5 ml	
	10% SDS	2.5 ml	
	0,5 M EDTA		10 ml
	H_2O	35 ml	
	total volume	50 ml	
	sterilising by autoclaving		
1xM9 medium	Na_2HPO_4	6 g	
	KH_2PO_4	3 g	
	NH_4Cl	1 g	
	NaCl	0,5 g	
	adjust with H_2O to	1000 ml	
	sterilising by autoclaving		
5x M63 salts	$(NH_4)_2SO_4$	10 g	
	KH_2PO_4	68 g	
	adjust with H_2O to	800 ml	
	adjust pH to 7.0		

	sterilising by autoclaving		
McConkey agar	BD (Franklin Lakes, NJ, USA)		
McConkey plates	McConkey agar	10 g	
	adjust with H_2O to	225 ml	
	sterilising by autoclaving		
	add 25 Galactose	25 ml	
	add appropriate antibiotics		
Methylcellulose	Carboxymethylcellulose		3.75 g
	adjust with H_2O to		232 ml
	sterilising by autoclaving		
	add:		
	FCS		25 ml
	2x DMEM		232 ml
	Glutamine		5 ml
	non-essential aminoacids		2.5 ml
	Penicillin/Streptomycin		5 ml
Minimal plates containing 2-deoxy-galactose (DOG) and CAM	agar	4 g	
	adjust with H_2O to	200 ml	
	sterilising by autoclaving		
	add:		
	5x M63 salts	50 ml	
	1 M $MgSO_4$	0.5 ml	
	D-biotin	1.25 ml	
	L-leucin	1.1 ml	
	10% glycerol	5 ml	
	10% DOG	5 ml	
	CAM	20 µg/ml	
solid medium 1.5% agar	BD (Franklin Lakes, NJ, USA)		
15% sucrose-virus standard buffer (VSB)	sucrose	75 g	
	adjust with VSB to	500 ml	
	sterilising by autoclaving		
10x TRIS-Borate-EDTA (TBE) buffer	TRIS		108 g
	Boride acid		55 g
	adjust with H_2O to		900 ml
	0.5 M Na_2EDTA (pH 8)		40 ml
	adjust pH to 8.0		

	adjust with H_2O to	1000 ml
	sterilising by autoclaving	
TE-buffer	1 M TRIS (pH 8)	10 ml
	Na2EDTA (pH 8)	200 µl
	adjust with H_2O to	800 ml
	adjust pH to 8.0	
	adjust with H_2O to	1000 ml
	sterilising by autoclaving	
VS Buffer	TRIS/HCl	6.055 g
	KCl	0.895 g
	EDTA (Na_2 salt)	1.86 g
	adjust with H_2O to	800 ml
	adjust pH to 7.8 with HCl	
	adjust with H_2O to	1000 ml
	sterilising by autoclaving	

2.5 Kits, Size Standards and Enzymes

The kits used to isolate Plasmids or BACs from bacteria, and DNA from polymerase chain reaction (PCR) samples or agarose gels were obtained from Qiagen (Hilden, DE): “QIAGEN Plasmid Mini Kit”, “QIAGEN Plasmid Maxi Kit”, “QIAGEN Large-Construct Kit”, “QIAquick Gel Extraction Kit” and “QIAquick PCR Purification Kit”. To isolate viral DNA the “High Pure Viral Nucleic Acid Kit” from Roche (Mannheim, DE) was used. Restriction enzymes were obtained from NEB (Ipswich, MA, USA). Proteinase K and size standards “GeneRuler 1 kb DNA Ladder Plus” and “GeneRuler 1 kb DNA Ladder” were obtained from Fermentas (Thermo Fisher Scientific, Waltham, MA, USA).Thermostable polymerases were obtained from NEB (MA, USA) and Bioline (Luckenwalde, DE).

2.6 Peptides/ Antibodies/ Dextramer

2.6.1 Peptides

Peptides were synthesized and HPLC purified (65 – 95% purity) in the research group "Peptide Synthesis" of Werner Tegge at the Helmholtz Centre for Infection Research (HZI).

ORF	Haplotype	Sequence
IE3 (MCMV)	K^b	^{416}RALEYKNL^{423}
M45 (MCMV)	D^b	^{985}HGIRNASFI^{993}
M38 (MCMV)	K^b	^{316}SSPPMFRV^{323}
gB (HSV-1)	K^b	^{498}SSIEFARL^{505}

2.6.2 Anti-Mouse Antibodies

Antibody	Fluorochrom	Company
anti-CD3	Allophycocyanin-eFluor780	clone 17A2; eBioscience, San Diego, CA
anti-CD4	Pacific Blue	clone GK1.5; Biolegend, San Diego, CA
anti-CD8a	PerCP-Cy5.5	clone 53-6.7; Biolegend
anti-CD44	AlexaFluor700	clone IM7; Biolegend
anti-CD62L	eFluor 605NC	clone MEL-14; eBioscience
anti-CD127	PE	clone A7R34; Biolegend
anti–IFNγ	Allophycocyanin	clone XMG1.2; Biolegend
anti–TNFα	FITC	clone MP6-XT22; Biolegend
CD49d purified		clone 37.51; eBioscience

CD28 purified		clone R1-2; eBioscience
anti-CD8	eFluor 450	clone 53-6.7, eBioscience
anti-CD62L	PE	MEL-14, BD
anti-CD44	FITC	IM7, BD
Annexin V	Allophycocyanin	BD
7AAD (7-Amino-actinomycin D)	(PerCP)	Sigma-Aldrich

2.6.3 Dextramer

Allophycocyanin labelled MHC Dextramer, H-2K^{b} RALEYKNL (Immudex, Kopenhagen, DK)

2.7 Materials and Equipment

Accuri Cytometer	BD (Franklin Lakes, NJ, USA)
Beakers	Nalgene (Rochester, NY, USA)
Centrifuge Tubes for Blood Samples	FisherBrand (Thermo Fisher Scientific, Waltham, MA, USA)
Butterfly Needle	Dispomed Witt (Gelnhausen, DE)
Cell Culture Dishes	Greiner Bio-One (Frickenhausen, DE)
Cell Culture Flasks	Thermo, nunc (Thermo Fisher Scientific, Waltham, MA, USA)
Cell Culture Plates	BD (Franklin Lakes, NJ, USA)
Cell Scrapers	TPP (St. Louis, MO, USA)
Cell strainer	BD (Franklin Lakes, NJ, USA)
Centrifuges	Thermo (Thermo Fisher Scientific, Waltham, MA, USA), eppendorf (Hamburg, DE) and sorvall (Thermo Fisher Scientific, Waltham, MA, USA)
15 ml/ 50 ml Centrifugation	Greiner Bio-One (Frickenhausen, DE)

Tubes	
Corex Tubes	GENcompare (Brüssel, BE)
Cryo Tubes	Brand (Wertheim, DE)
Cuvettes	Sarstedt (Nümbrecht, DE)
Douncer	Kleinfeld (Gehrden, DE)
Electrophoresis Systems	Serva (Heidelberg, DE) and Biozym (Hessisch Oldendorf, DE)
Folded Filter	Schleicher & Schuell (Dassel, DE)
Gene Pulser II	Bio-Rad (München, DE)
Gene Pulser Cuvette 0.2 cm	Bio-Rad (München, DE)
Incubators	Thermo (Thermo Fisher Scientific, Waltham, MA, USA) and NuAire (Plymouth, MN, USA)
Long Size Pipettes	WU Mainz (Mainz, DE)
LSRII	BD (Franklin Lakes, NJ, USA)
LSR Fortessa	BD (Franklin Lakes, NJ, USA)
Micro Tubes	Biozym (Hessisch Oldendorf, DE) and Greiner Bio-One (Frickenhausen, DE)
Pipettes For Cell Culture	Greiner Bio-One (Frickenhausen, DE)
Pipette Tips	STARLAB (Hamburg, DE)
Plates for Cultivation of Bacteria	Nerbe-plus (Winsen/Luhe, DE)
Safety Work Bench	Thermo (Thermo Fisher Scientific, Waltham, MA, USA)
Scalpels	Braun (Melsungen, DE)
Small FACS Tubes	Micronic (Lelystad, NE)
Software	CFlow Plus from BD, Version 1.0.227.4 FlowJo from Tree Star (Ashland, OR, USA) Version 9.4 FACSDiva from BD, Version 6.1 GraphPad Prism from GraphPad (La Jolla, CA,USA), Version 5.04 Vector NTI Advance from life technologies (Carlsbad, CA,USA), Version 11.0
SpectroPhotometer ND-1000	peqlab biotechnologie (Erlangen, DE)
SpectroPhotometer UltraSpec 3100 pro	GE Healthcare (Thermo Fisher Scientific, Waltham, MA, USA)
Syringes	Braun (Melsungen, DE)

Thermocycler	life technologies (Carlsbad, CA,USA)
Thermomixer	Eppendorf (Hamburg, DE)
Ultracentrifuge	Thermo (Thermo Fisher Scientific, Waltham, MA, USA)
Ultraviolet (UV) Transilluminator	Biometra (Göttingen, DE)
Waterbath	GFL (Burgwedel, DE)

2.8 Mice

C57BL/6	Janvier (Le Genest Saint Isle, France)
C57BL/6 R26Cre.ER^{T2}	central breeding facility of the HZI, Braunschweig
C57BL/6 Rag2^{flox}/^{flox}xR26Cre.ER^{T2}	central breeding facility of the HZI, Braunschweig
Balb/c CMV-Cre	central breeding facility of the HZI, Braunschweig

2.9 Mice Handling

All animal experiments performed at HZI were performed in compliance with the German animal protection law (TierSchG BGBI S. 1105; 25.05.1998) and were approved by the responsible state office (Lower Saxony State Office of Consumer Protection and Food Safety). The mice were housed and handled in accordance with good animal practice as defined by FELASA and the national animal welfare body GV-SOLAS. If necessary the transgenic mice were genotyped as described in the appendix.

2.10 Cell Lines

M2-10B4	CRL-197; American type culture collection ATCC, VA, USA
NIH 3T3	CRL-1658; American type culture collection ATCC, VA, USA
C57BL/6 MEFs	all primary cells have been isolated at HZI, Braunschweig
Balb/c MEFs	all primary cells have been isolated at HZI, Braunschweig
Balb/c CMV-Cre MEFs	all primary cells have been isolated at HZI, Braunschweig
C57BL/6 R26Cre.ER^{T2} MEFs	all primary cells have been isolated at HZI, Braunschweig

2.11 Viruses

The MCMV genome was previously cloned as a BAC [189], and the corresponding MCMV WT virus has been characterized in detail [190].

The recombinant MCMV IE1/3^{flox} was generated by 2-step BAC recombineering using the galactokinase (*galK)* selection, predominantly as described before [191] and in “Viral Mutagenesis” on page 38. The virus MCMV IE2^{SL} was provided by Iryna Dekhtiarenko (HZI, Braunschweig) and described in Dekhtiarenko *et al.* [155].

2.12 Bacteria

The *E. coli* strain which carries the MCMV BAC and has been used for the viral mutagenesis was described previously [191].

strain	Genotype	Phenotype
SW102	*mcrA Δ(mrr-hsdRMS-mcrBC) ΔlacX74 deoR endA1 araD139 Δ(ara, leu) 7697 rpsL recA1 nupG* ϕ80d*lacZΔM15 [λc*1857 *(cro-bioA)<>Tet] gal*$^{+}$ *ΔgalK*	Gal^{-}

2.13 Molecular Biology

2.13.1 Cultivation and Storage of *Escherichia coli*

The *E. coli* cells were cultivated in LB medium [192]. The medium was supplemented with the appropriate antibiotic (final concentrations for different antibiotics see page 24) for bacteria, which contains plasmids or BACs with an antibiotic-resistance-cassette to sustain the selection pressure on the appropriate plasmid or BAC. For solid medium, 1.5% agar was added to the liquid medium. The cells were incubated at 37℃ whereas cells containing the strain SW102 were incubated at 32℃, on LB agar plates or as a liquid culture with shaking at 120 revolutions per minute (rpm). These rpm was used in all subsequent methods for cultivation of *E. coli*.

For long term storage, the *E. coli* cells were incubated overnight (ON) in LB medium containing the appropriate antibiotic at a suitable temperature with shaking. 750 µl of the ON bacterial culture were mixed with 250 µl 80% Glycerol (3:1) in a cryo vial and stored at -70℃.

2.13.2 Isolation of Plasmid-DNA from *E. coli*

E. coli strains containing the desired plasmids were incubated ON in 5 ml LB medium and the appropriate antibiotic at 37℃, with shaking. Isolation of plasmid DNA was performed using the QIAGEN Plasmid Mini Kit according to the manufacturer's protocol. The concentration of DNA was determined photometrically at 260 nm with the SpectroPhotometer ND-1000.

2.13.3 Isolation of BAC-DNA from *E. coli*

E. coli strain SW102 containing the corresponding BAC was incubated ON in 5 ml LB medium with the appropriate antibiotics at 32℃ with shaking. This starter culture was diluted in 250 ml LB-medium with the suitable antibiotics and incubated ON at 32℃ with shaking. For isolation of BAC DNA, the Qiagen Plasmid Maxi Kit (QIAGEN-tip 500) with modified protocol was used. To isolate BAC DNA for restriction analysis, the Qiagen Large-Construct Kit was used.

In brief, bacteria from the main culture were isolated and the pellet was re-suspended in P1 buffer by pipetting up and down. To lyse the cells, P2 lysis buffer was added and the suspension was mixed by gently inverting the tubes 20 times, to prevent shearing of the genomic DNA. The lysis was stopped after 5 minutes (min) by adding chilled

buffer P3. The suspension was mixed by gently inverting the tubes 20 times and incubated for 30 min on ice to precipitate the DNA. The proteins and cell debris were removed by two centrifugation steps. To prevent clogging of the QIAGEN-tips, the supernatant (SN) was filtered over a prewetted and folded filter into new tubes. The DNA was precipitated with 0.7 volume of Isopropanol and centrifuged at 20000 g for 30 min at 4℃. Before using the QIAGEN-tip, it was equilibrated with QBT buffer. The DNA pellet was re-dissolved in 500 µl TE-buffer and re-suspended in 11.5 ml QBT buffer. To separate the BAC DNA from the genomic DNA, the SN was added to the column and washed twice with QC buffer. To elute the DNA from the column into Corex tubes, prewarmed (65℃) QF buffer was added. To increase the yield of DNA, a second precipitation step was performed. The eluate was centrifuged at 15000 g for 30 min at 4℃ and then was washed with 70% Ethanol (EtOH). To remove the EtOH from the DNA, the pellet was air-dried for 5 - 10 min. The DNA was re-suspended in 50 - 150 µl TE buffer ON and stored at 4℃.

The concentration of the DNA was determined by photometric analysis at 260 nm in a SpectroPhotometer ND-1000.

2.13.4 Isolation of Genomic DNA from Mouse Cells for Genotyping

To genotype the transgenic mice, a tip of the mouse tail was cut and lysed in 498 µl lysis buffer and 2,5 µl Proteinase K at 56℃ with s haking at 800 rpm for at least 6 hours (h). To inactivate the Proteinase K, the lysate was incubated for 5 min at 95℃. To remove proteins and cell debris a centrifugation step at 16000 g for 5 min was performed in a table microcentrifuge. The SN, containing the DNA, was transferred to a new tube and the DNA was purified by Isopropanol/EtOH-precipitation. To this end, 500 µl Isopropanol were added to the lysate and tubes were inverted slowly until both phases were mixed. After centrifugation at 16000 g for 10 min, the pellet was washed with 500 µl 70% EtOH, incubated for 10 min at room temperature (RT) and centrifuged at 16000 g for 5 min. The pellet was dried at RT or at 37℃ until all EtOH was evaporated. The DNA was re-suspended in 200 µl TE-buffer at 37℃ for 10 min with shaking at 450 rpm. The genomic DNA was stored at 4℃.

2.13.5 Polymerase Chain Reaction (PCR)

The PCR was used to amplify particular DNA fragments [193], [194]. The reactions were done with a thermo stable polymerase and oligonucleotides (primers). The primers were used in a final concentration of 1 pmol in reaction buffer according to the manufacturer's instructions of the used polymerase. The nature and duration of the temperature profile was determined by the manufacturer, the used primers and the template. The approaches and programs which were used in this thesis can be found in the appendix. To verify the amplified fragments, the PCR products were applied together with a suitable DNA size standard on a 1% agarose gel, which was prepared with 0.5 x TBE buffer.

2.13.6 Enzymatic Digestion of DNA with Restriction Endonucleases

For analytical restriction reactions 400 ng of plasmid DNA or 0.75 µg of BAC DNA were digested with 2 units of the restriction enzyme. The sample, containing plasmid DNA, was incubated in a volume of 10 µl for 1 h at 37℃. The sample, containing BAC DNA, was incubated in a volume of 40 µl for 3 h at 37℃. The reaction buffers were used as specified by the manufacturer. For the restriction of preparative amounts of DNA, larger volumes and amounts of enzymes were used.

2.13.7 Separation of DNA in an Agarose Gel

For the agarose gel 0.5 x TBE buffer was boiled with 1% agarose. For gels, analysing BAC DNA restrictions with, 0.8% agarose were used. After cooling down, 0.005% GelRed was added to the viscous solution which was poured into the gel chamber. As running buffer 0.5 x TBE buffer was used. If necessary, a loading buffer was added 1:6 to the DNA sample before separation. The electrophoretic separation was done at 135 volt (V) DC (Direct Current) for 25 - 35 min for PCR products and at 80 V DC for 15 - 25 h to separate restriction fragments of BAC DNA. The DNA was visualised by irradiating the gel with UV light λ = 312/319 nm. To determine the size of the DNA fragments the "™ GeneRuler 1 kb DNA Ladder" or the "™ GeneRuler 1 kb Plus DNA Ladder" were used as size standards.

2.13.8 Isolation of DNA from an Agarose Gel

The DNA, stained with GelRed, was detected by a UV transilluminator at λ = 312/319 nm. The gel fragment, containing the desired band, was cut out with a scalpel and transferred into a 1.5 ml tube. The isolation of the DNA fragments was done with the QIAquick Gel Extraction Kit, according to the manufacturers' protocol. The DNA was eluted with 15 µl elution buffer and stored at -20 °C.

2.13.9 DNA Sequencing

The sequencing of the recombinant MCMV IE1/3^{flox} BAC and the PCR products was performed by the group "Genomanalytik" of Robert Geffers at the HZI.

2.13.10 Preparation of Electro-Competent *E.coli*

Electro-competence of *E. coli* cells requires that they are in the growing phase and clean of salt. Therefore a starter culture was done by growing bacteria ON in a 5 ml LB culture with the appropriate antibiotics at 37°C with shaking. 4 ml of the starter culture was diluted in 200 ml LB medium with the appropriate antibiotics and incubated for 3 - 4 h at 32°C with shaking. The optical density (OD) at 600 nm was measured with the UltraSpec 3100 pro at regular time intervals, until an OD of 0.5 – 0.6 was reached. The bacteria were pelleted at 2500 – 3500 g for 10 min at 1°C. To remove the salt from the bacteria, they were washed with 10% Glycerol. The following steps were performed on ice. The pellet was re-suspended in 10 ml chilled 10% Glycerol, which was added to the pellet with a chilled and sterile plastic pipette. The bacteria were pelleted at 2500 - 3500 g for 10 min at 1°C. The washing steps were repeated two times. After the last washing step the bacteria were re-suspended in the fluid from the sides of the tubes which reaches the bottom of the beaker. The bacteria were either aliquoted at 60 µl in chilled tubes or used immediately for transformation. For storage at -70°C, they were shock frozen in liquid N_2.

2.13.1 Transformation of *E.coli* with Electroporation

To transform DNA into *E. coli*, the bacteria cell membranes were made permeable for molecules of high molecular weight by short electrical pulses of high field strength. Therefore, electro competent bacteria cells were thawed on ice, up to 12 µl PCR product was added, mixed carefully and incubated for 2 min on ice. During this time, the DNA could adhere to the bacterial cells. The mixture was transferred into a chilled cuvette without air bubbles. For the electroporation the "Bio-Rad gene pulser II" was used.

The electroporation was done for 3.5 – 5 milliseconds (msec) with:

Capacity of the condenser = 25 µF

Electric tension U = 2.5 kV

Resistance R = 200 Ω

Immediately after electroporation the bacterial cells were transferred to a glass tube containing 1 ml LB medium without antibiotics. The cells were incubated for 1 h at 37℃ with shaking to give them time to produce resistance to the selective antibiotics. From the bacterial culture 10 µl, 100 µl and 200 µl were seeded on LB agar plates supplemented with the appropriate antibiotic and incubated ON at 37℃ until bacterial colonies have been formed.

2.13.2 Viral Mutagenesis: Recombinant BAC MCMV IE1/3^{flox}

The recombinant virus MCMV IE1/3^{flox} was generated using the *galK* positive/counterselection described by Warming *et al*. [191], with modifications described by Dölken *et al*. [195].

For the recombinant MCMV, the BAC “MCMV-C3X m129repair” transformed in *E. coli* strain SW102 was used [191]. The bacterial strain SW102 contains a stable integrated defective λ prophage. From its strong promoter *p*L, the genes *exo*, *bet* and *gam* are expressed under the control of the temperature-sensitive repressor *c1857*. These genes are responsible for the recombination of linear fragments into the BAC genome [196], [197], but are not expressed at 32℃. To act ivate them, an incubation step at 42℃ for at least 15 min is necessary. Furthermore the SW102 cells contain the galactose operon, except for the galactokinase gene for the *galK*, and hence cannot metabolise galactose, unless the *galK* is expressed *in trans*.

In the recombinant MCMV IE1/3^{flox} the ORFs of the immediate-early genes *ie1* and *ie3* were flanked by two *loxP* sequences (Figure 8), to allow its target, the enzyme Cre recombinase, to recombine the MCMV genome.

13 bp 8 bp 13 bp

ATAACTTCGTATA – ATGTATGC – TATACGAAGTTAT

Figure 8 Sequence of the *loxP* site.

LoxP is a DNA sequence derived from the bacteriophage P1, which is composed of 34 bp. It consists of an asymmetric 8 bp sequence which is flanked by two palindromic sequences of 13 bp.

The first *loxP* sequence was inserted between the stop-codon of *ie3* and its poly(A) tail, replacing the nucleotides 177965 – 177974 [37], where it does not affect the correct reading of the genes. For the second *loxP* sequence, the non-coding Exon 1 of *ie1/3* was chosen, replacing the nucleotides 182837 – 182846 [37] (Figure 9). The location is far enough away from the start-codon, not to disturb the correct reading of the RNA-polymerase or of the ribosome and on the other hand the promoter-enhancer structure of *ie1/3* is not impaired. Another advantage of this strategy is that the recombination does not remove the promoter-enhancer from the viral genome, and therefore the genes *ie1* and *ie3* cannot be expressed ectopically.

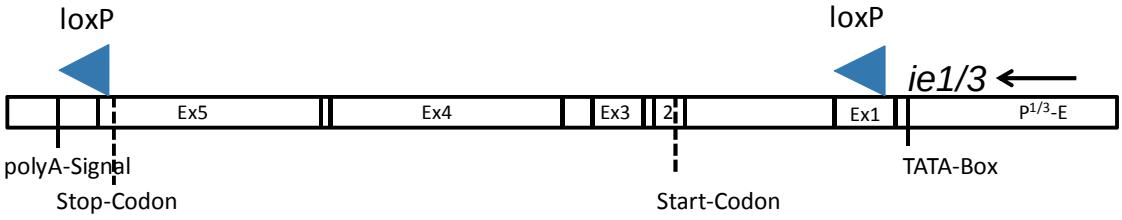

Figure 9 Overview about the modified *ie1/3* locus of MCMV IE1/3^{flox}.

The left *loxP* sequence was inserted into the non-coding Exon 1 of *ie1/3*. The right *loxP* sequence was inserted in the Exon 5 between the stop-codon and the poly(A)-tail of *ie3*.

In a first step, a linear PCR product was generated in a reaction with the pGPS/galKn plasmid [195] as template. The *galK/Kn* cassette, containing the *galK* and the *Kan* genes, was amplified with primers which carried at their 5' end 50 bp homology sequences to the site in the viral genome at which the *loxP* sequence was introduced and homologies to the *galKn* plasmid at their 3' end. The primers P9 and P11 were used to amplify the *galK/Kn* cassette for the insertion between the stop-codon of *ie3* and the poly(A) tail, and the primers P43 and P45 were used to amplify the *galK/Kn* cassette for the insertion into the non-coding Exon 1 of *ie1/3*. The PCR programs and reagents used are shown in the appendix. To remove the template DNA from the PCR

reactions, a digestion with 20 units of the enzyme *DpnI* was performed at 37℃ ON, which was followed by a purification step, in which the PCR product was extracted from a 1% agarose gel (see page 37). The transformation was done into electro competent SW102 bacteria containing the WT BAC as described above (see page 37), with the following modifications. In all steps, the bacterial cells were incubated at 32℃ instead of 37℃. To activate the genes *exo*, *bet* and *gam*, the bacterial cells were incubated at 42℃ for 15 min with shaking prior to induce electr o-competence (page 37). The electro-competent *E. coli* were transformed with the PCR products according to the protocol on page 38. 10 µl, 100 µl and 500 µl of the transformed bacterial culture were seeded on Kn/CAM agar plates. Clones that integrated the product were selected from Kn/CAM plates after 1 – 2 days of incubation and confirmed by monitoring on McConkey agar plates, restriction pattern analysis and sequencing.

In a second mutagenesis step, a PCR product carrying the *loxP* sequence, flanked by MCMV sequences, was introduced into the MCMV BAC by homologous recombination and metabolic selection on minimal 2-deoxy-galactose plates. The construct for introducing the *loxP* sequence into the 3' end of *ie3*, was synthesised by GENEART (Regensburg, DE) and the primers P18 and P19 were used to amplify the construct. The construct, used to introduce the *loxP* sequence into the Exon 1 of *ie1*/3, was synthesised by Eurofins MWG Operon and the primers P46 and P47 were used to amplify the construct. The PCR programs and reagents used can be found in the appendix. The obtained PCR products were handled and transformed into the *E. coli* as described above, with the following modifications. After transformation, the bacteria were incubated in 10 ml LB medium for 4.5 h at 32℃ and washed twice with 1 x M9 medium to get rid of the LB medium. 1 ml of the bacterial culture was centrifuged at 16000 g for 15 seconds (sec) at RT and re-suspended in 1 ml 1 x M9 medium. 10 µl, 100 µl and 500 µl were seeded on Minimal plates containing DOG and CAM. Clones that integrated the product were selected from the Minimal plates after 4 – 5 days of incubation and confirmed by restriction pattern analysis and sequencing.

The correct insertion of the *loxP* sites into the BAC and the absence of undesired mutations were confirmed by sequencing of the entire BAC. The newly generated BAC was used for transfection of murine embryonic fibroblasts (MEFs) to generate infectious viral progeny (see page 43).

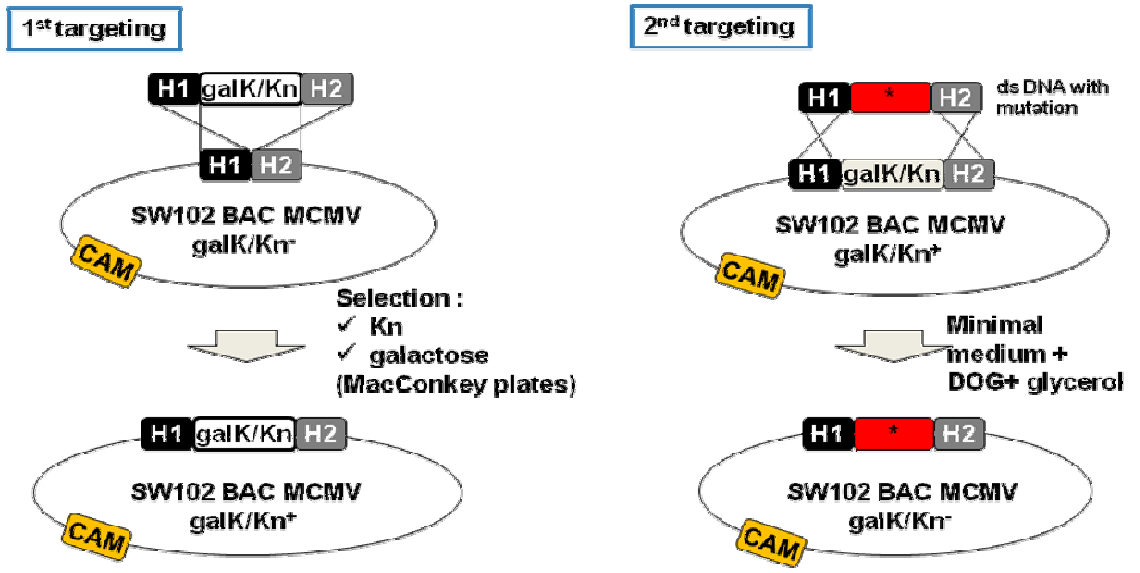

Figure 10 Overview *galK/Kn* selection scheme.

In the first step, the *galK/Kn* cassette, flanked with homologies to the target site of MCMV BAC, was introduced. The selection on bacteria, which were positive for the *galK/Kn* cassette, was done by using MacConkey agar plates supplemented with kanamycin and galactose. In the second step, the construct, which carried the desired mutation and was flanked with the same homologies as in the first step, was inserted. The selection was performed on minimal plates, supplemented with DOG and glycerol for bacteria which were negative for the *galK*, but positive for the desired mutation. (modified from [191])

2.14 Cell Culture

2.14.1 Cultivation of Mammalian Cells

The cells were kept in a cell culture incubator at 37℃ and with 5% CO_2. For most of the cell types, the used cell culture medium was DMEM supplemented with 10% FCS, 1% Glutamine and 1% Penicillin/Streptomycin (suppl. DMEM).

To maintain the cells, they were trypsinised and passaged on new plates. The medium was removed from the confluent cells by vacuum aspiration, followed by a wash with PBS to get rid of residual medium. To detach the cells from the plastic surface, they were trypsinised at 37℃ for approximately 5 min. T he reaction was stopped by adding medium containing 10% FCS. The medium was removed from the cells by centrifugation and then the cells were re-suspended in an appropriate amount of medium and seeded to a new flask or dish.

2.14.2 Storage of Mammalian Cells

Cells were incubated either on a 14.5 cm ∅ dish or a T175 flask until they reached 80 - 90% confluence. The freezing medium, containing 10% DMSO and 90% FCS was kept on ice all the time. The cells were washed once with PBS, to remove medium and FCS. To detach cells from the surface, there were treated with trypsin and incubated at 37℃ for approximately 5 min. To inactivate the try psin, medium with 10% FCS was given to the cells and the suspension was collected in a blue cap tube. The trypsin was removed from the cells by centrifugation and the cells were re-suspended in freezing medium (3 ml freezing medium per dish or flask). The cells were aliquoted to 1 ml in pre-chilled cryo vials. To freeze the cells slowly, they were put in a container, which was surrounded with Isopropanol and frozen at -20℃ for 2 days, before they were frozen in liquid nitrogen.

2.14.3 Preparation of Mouse Embryonic Fibroblasts (MEFs)

MEFs were prepared as described before [198] with some modifications. At day 13 - 15 after the positive plaque-check, the pregnant mouse was sacrificed by CO_2 asphyxia, sterilized with EtOH and fixed on a Styrofoam board. The abdomen was opened with sterile instruments and the uterus with the foetuses was extracted and transferred to a dish with PBS. The foetuses were isolated from amnion and placenta and transferred to a clean dish with PBS to wash away the blood and remains of other tissue. From liver and intestine as much as possible was removed and the foetuses were transferred to another dish without PBS to mince them into very small bits using scalpels. 10 ml trypsin per foetus were added and up to 7 foetuses were transferred to an Erlenmeyer beaker filled with beads of glass. The cells were separated by stirring the cell suspension on a magnetic stirrer with a magnetic bar for 1 h at 37℃, which moves the beads around, but not too vigorously. Then, an equal volume of suppl. DMEM was added to the cells to make the suspension less viscous, upon which the cells were transferred to 50 ml tubes. To get rid of the trypsin, the cells were pelleted by centrifugation at 314 g for 10 min at RT. The SN was removed carefully and the cells were re-suspended in suppl. DMEM. To get rid of big tissues bits the cells were passed through a 100 µm cell strainer and pooled afterwards. The cells were counted in a Neubauer's chamber and cultured in T175 flasks with an amount of 10^7 cells per flask using suppl. DMEM. After one day of incubation, the cells were washed twice with PBS and fresh suppl. DMEM was added. When the cells reached 90% of confluence, they were expanded 1/3 on T175 flasks. To detach the cells from the surface, there were

treated with trypsin and incubated for approximately 5 min at 37℃. To inactivate the trypsin, suppl. DMEM was given to the cells and the suspension was collected in a 50 ml tube. To remove the trypsin from the cells, there were centrifuged for 5 min at 314 g. The SN was discarded and the cells were re-suspended in a proper amount of suppl. DMEM and cultured in T175 flasks. The cells were incubated until they reached 80% of confluence, in order to be able to freeze them in the growing phase. The freezing medium, containing 10% DMSO and 90% FCS was kept on ice all the time. The cells were washed once with PBS, to remove medium and FCS. The cells were detached from the surface like described above. The pelleted cells were re-suspended in freezing medium (3 ml freezing medium per T175 flask). The cells were aliquoted to 1 ml in pre-chilled cryo vials. To freeze the cells slowly, they were put in a container, which was surrounded with Isopropanol and frozen at -20℃ for 2 days, before they were frozen in liquid nitrogen.

2.14.4 Generation of MCMV from BAC DNA by Transfection of Mammalian Cells

To generate MCMV from BAC DNA, the DNA was transfected into mammalian cells. For the transfection, freshly prepared MEFs were used. One day before transfection the MEFs were seeded on a 6-Well plate, with 6 x 10^5 cells per well in suppl. DMEM. On the day of transfection they were 50 - 60% confluent.

Transfection was performed with the Fugene HD Transfection Reagent, MCMV BAC DNA and Opti-MEM I Reduced Serum Medium, all warmed to RT prior to the procedure beginning. 2 µg of the BAC DNA (260/280 nm: ca. 1.8) were mixed with Opti-MEM in a total volume of 100 µl. 4 - 8 µl FUGENE Reagent were vortexed and added to the diluted DNA without touching the plastic. The solution was mixed by pipetting up and down. To allow the FUGENE Reagent to attach to the DNA, it was incubated at RT for at least 15 min. Transfection was optimised by replacing the standard cell culture medium with DMEM and 10% FCS, but without antibiotics. 100 µl of the transfection complex was added to the cells in a drop-wise manner and swirled, to allow its distribution over the entire plate surface. Cells were incubated with the complex at 37℃ for 5 h, upon which the medium with the transfection complex was replaced with fresh suppl. DMEM and incubated for 48 - 72 h. To allow cells to grow, they were split into a T25 flask.

The transcription of viral genes initiated from the BAC DNA and after a week later, virus growth could be observed by the presence of viral plaques, consisting of a clearance in

the cell monolayer surrounded by rounded cells. Cell rounding was caused by virus induced morphological changes in the cell, which are also termed “cytopathic effects” [199]. The newly generated viruses initially still carried the BAC vector in their genome. The BAC vector allows the BAC DNA replication in *E. coli* and carries an antibiotic selection marker, ensuring that only cells carrying the BAC, replicate in the presence of CAM. Its presence in the replicating virus results in a genome over length, which impairs DNA packaging into the virion and thus deters viral replication. Hence, in order to obtain a virus which is modified as little as possible, the vector was removed. Since the vector is flanked by homologous sequences of the MCMV genome, the recombination of homologous sequences can lead to the excision of the BAC sequence. Selection pressure favours the viruses which have lost the BAC. Therefore, consecutive passaging of the reactivated virus results in an enrichment of the MCMV WT genome which have lost the BAC [26].

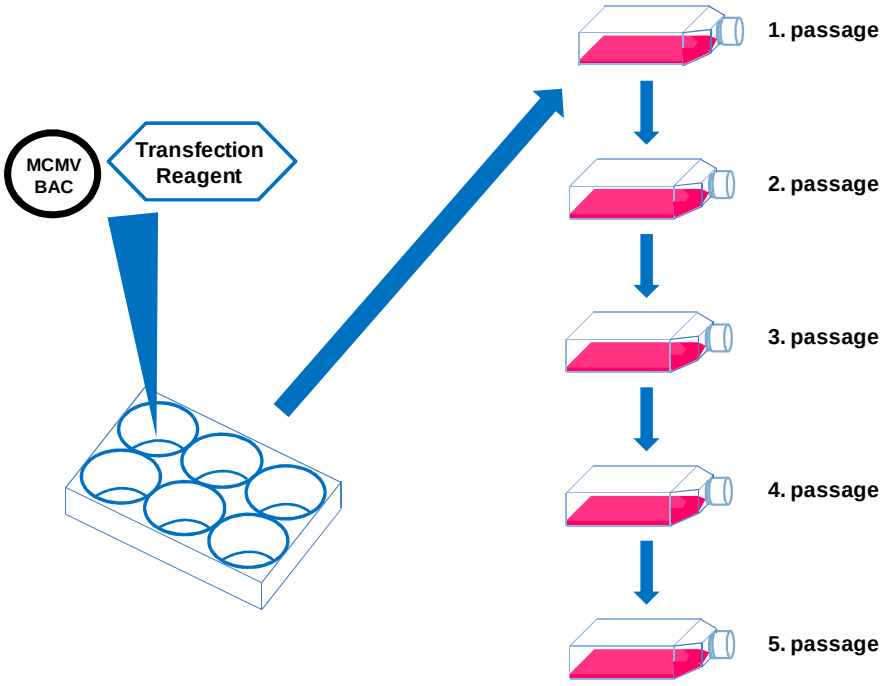

Figure 11 Generation of MCMV by transfection and passaging.

MEFs were transfected with the MCMV BAC DNA in presence of a transfection reagent. To get rid of the BAC vector, the virus was passaged five times on M2-10B4 cells.

To get rid of the BAC cassette, the virus was passaged five times. After the transfected cells showed 100% cytopathic effects, SN containing the MCMV virus was used to infect a new monolayer of mouse bone marrow stromal cells (M2-10B4 cells). Thus, 1 ml SN of the infected MEFs was used to infect one T25 flask of M2-10B4 cells. When these cells were lysed, 1 ml SN was used to infect another T25 flask of M2-10B4 cells,

which was pursuit until the 5th passage. Then, the SN of the completely infected and lysed cells was used for preparing a virus stock (Figure 11).

2.14.5 Preparation of Virus Stock

Viruses were propagated as described before [200] with some modifications.

Two 15 cm plates with M2-10B4 cells at 30 - 50% confluence were each infected with 1 ml SN from passage 5 of virus after transfection. After 4 - 5 days, cells and SN were harvested. The suspension was used directly to infect cells for the virus stock or it was frozen at -70°C before usage.

M2-10B4 cells from fourteen 15 cm plates were 90 - 100% confluent, when they were used to make the virus stock. The medium was removed from the plates and the cells were washed with PBS. To detach the cells, they were incubated in the presence of trypsin at 37°C for approximately 5 min. To stop the reaction, suppl. DMEM was added and the cells were transferred to four 50 ml centrifuge tubes and centrifuged at 314 g for 5 min at RT. Afterwards the SN was removed and the cells were re-suspended in 10 ml suppl. DMEM per centrifuge tube. The cells from the centrifuge tubes were transferred in two 500 ml bottles and medium was added to a volume of 400 ml per bottle. The suspension from the two plates infected in advance was added and mixed by inverting the flasks. The cells were seeded to forty 15 cm plates (20 ml cell suspension per plate) and incubated for 4 – 5 days.

The virus was harvested once most of the cells were lysed and detached from the bottom. Therefore, most of the SN was transferred to 400 ml centrifuge bottles on ice, but 3 – 4 ml was left on the plates. The cells were scraped from the plates with a cell scraper and collected with the medium left on the plate. The cells were pelleted by centrifugation at 5800 g for 15 min at 4°C. The SN was collected and stored on ice. To obtain virus from the cells the pellet was dounced in 4 ml suppl. DMEM in prechilled 7 ml douncers. To remove the cell debris, the re-suspended pellet was centrifuged at 17000 g for 10 min at 4°C. The SN, containing the virus, was added to the SN obtained in the first centrifugation round, and the virus was pelleted by centrifugation at 27600 g for 3.5 h at 4°C. 4 ml of suppl. DMEM was added to the pellet and it was softened on ice ON at 4°C, before douncing in a prechilled douncer. To purify the virus, the re-suspended pellet was put carefully on top of 10 ml cold 15% sucrose-VSB. The suspension was overlayed with VSB without mixing the layers. The virus was obtained by centrifugation at 72000 g for 1 h and 20 min at 4°C in an Ultracentrifuge. To soften the pellet, it was incubated in 1.2 ml VSB ON on ice at 4°C, before douncing in a prechilled douncer. The suspension was transferred to a 2 ml reaction tube and

centrifuged at 1200 g for 2 min at 4℃. The SN from that step was centrifuged again and the SN from the last step was aliquoted in 0.5 ml micro tubes at 30 – 50 µl and stored at -70℃ until quantification.

2.14.6 *In Vitro* Infection

Monolayers of NIH3T3 cells were infected in 24-well plates with a multiplicity of infection (MOI) of 0.1. The cells were incubated with the virus for 1 h and 37℃ and the plates were stirred gently from time to time. From one well, the inoculum was removed and stored at -70℃. From the other wells the mediu m was removed and the cells were washed once with PBS, before 1 ml suppl. DMEM was added to each well. The cells were incubated at 37℃ until the end of experiment. Every 24 h the SN was collected from one well for 6 days and stored at -70℃. The e xperiment was done in triplicate and the virus in the SNs was quantified on MEFs.

2.14.7 Infectious Virus Quantification

Tissue culture SN or virus stocks were titrated on MEFs by plaque assay [39] with modifications described previously [169].

The titration was done on primary MEFs, whose passage was not higher than 4 at the time of titration. The cells were cultivated in 10 cm plates in suppl. DMEM and when they reached 100% confluence, the cells were seeded on two 48-well plates and incubated at 37℃ ON. The virus was diluted from 10^{-1} to 10^{-9} in DMEM supplemented with 5% FCS on ice. Then the medium from the MEFs was removed and in triplicate wells replaced with 200 µl/well of the diluted virus (dilutions 10^{-2} to 10^{-9}). The plates were incubated for 1 h at 37℃. Afterwards, the vir us dilutions were removed from the cells and replaced with 500 µl/well prewarmed carboximethylcellulose and further incubated at 37℃ for 4 days before the read out.

2.14.8 *In Vitro* Recombination of MCMV IE1/3^{flox}

MEFs expressing inducible Cre.ER^{T2} (derived from transgenic C57BL/6 R26Cre.ER^{T2} mice) were seeded in 10 cm plates at 30 - 35% confluence and incubated for 48 h in presence of 1 µM Tam at 37°C. Cells from one 10 cm plate were transferred to 6 Wells of a 12-well plate and infected with MCMV IE1/3^{flox} at MOI 0.1. After incubating the cells for additional 48 h, the SN was harvested and the virus in 15 µl of SN was used as template to analyse the *ie1/3* gene locus for recombination by PCR with the primers P18 and P47. The programs and reagents used can be found in the appendix.

2.14.9 IFNβ-Induced Latency and Virus Reactivation

20.000 CMV-Cre MEFs/well (MEFs, expressing a Cre recombinase from the transgenic mice Balb/c CMV-Cre) were incubated in 96-well plates for 24 h in the presence of 500 or 100 units of IFNβ. The cells were washed once with PBS and infected with a MOI 0.001 or 0.0001 with MCMV WT or MCMV IE1/3^{flox}. The medium containing the virus was removed after 1 h at 37°C and replaced with suppl. DMEM containing IFNβ (suppl. DMEM without IFNβ in control wells) and incubated for 7 days. The medium containing the IFNβ was replaced every 2 – 3 days with fresh medium + IFNβ. At 7 days post infection (dpi) the IFNβ containing medium was replaced with normal medium in selected wells and the cells were incubated for additional 21 days. The wells were monitored for infectious plaques throughout the experiment.

2.15 Immunological Biology

2.15.1 *In Vivo* Infection

Mice were infected i.p. with a total volume of 200 µl of purified, tissue culture-derived MCMV diluted in PBS. The amount of input virus depended on the mouse strain: 2×10^5 plaque forming unit (pfu) were used to infect mice on the Balb/c background and 1×10^6 pfu were used for mice on C57BL/6 background.

2.15.2 Oral Administration of Tamoxifen

Mice were fed with pellets, containing 400 mg/kg Tam CRD Tam^{400}/CreER from Teklad for 2 - 4 weeks, depending on the experiment.

2.15.3 Virus Quantification in Organ Samples

Mice were euthanized by CO_2 asphyxia. The organs were dissected under sterile conditions and stored in 2 ml reaction tubes containing 1 ml RPMI at -70℃ until titration. If the whole organ was not collected, the reaction tubes containing 1 ml RPMI were weighed before and after the tissue collection.

The organs were titrated on MEFs. Cells from 10 cm plates with 100% confluence were seeded on two 48-well plates and incubated ON at 37℃. The organs were thawed on ice, homogenised on 100 µm-pore-size cell strainers and diluted in 5 ml RPMI. The dilutions of 1:10, 1:100 and 1:1000 were done in a total volume of 500 µl suppl. DMEM with 5% FCS. Then, the medium was removed and replaced with 200 µl/well of the homogenate dilutions. To allow the virus to reach the MEFs, the plates were centrifuged for 30 min in swing out buckets at 690 g, before they were incubated for additional 30 min at 37℃. Afterwards, the homogenate dilutions were removed from the cells and replaced with 500 µl/well of prewarmed carboximethylcellulose and further incubated at 37℃ for 4 days before the read out.

2.15.4 Peripheral Blood and Spleen Collection and Processing

Mice were anesthetized with Isofluran, before up to 150 µl blood were acquired from the retrobulbar venous plexus and diluted in 300 µl heparin in HBSS (2 units/ml). The blood samples were centrifuged and the erythrocytes were removed by brief osmotic shock lysis. Therefore, the tube was held on a vortex while 9 ml deionised water (dH_2O) were added, which was immediately followed by slow addition of 3 ml 4 x PBS to make the solution isotonic and stop the lysis. The tubes with the cells were kept on the vortex all of time, to ensure that the cells swirl the whole time. The samples were centrifuged again, but this time the SN was not removed completely. The cells were re-suspended in the rest of the SN (~ 100 µl) and transferred to a 96-well plate. The cells were pelleted by centrifugation and the excess fluid was removed by flicking the plate. Cells were detached from the bottom by briefly vortexing the plate and a second erythrocyte lysis was performed. 150 µl H_2O were added to the samples, followed by an addition of 50 µl 4 x PBS to stop the lysis. The plates were centrifuged, the excess volume flicked off and the cells used for fluorescent staining or peptide stimulation (see chapters 2.15.6 to 2.15.9).

To isolate splenic lymphocytes, mice were euthanized by CO_2 asphyxia and the spleen was stored in 2 ml tubes with 1 ml RPMI on ice until usage. The spleen was homogenized on 100 µm-pore-size cell strainers and diluted in 5 ml RPMI. The

erythrocytes were removed from the spleen samples by ACK lysis. The ACK buffer was added to the splenocytes and incubated for 2 min at RT, to allow the erythrocytes to lyse. The reaction was stopped by adding 2 volumes RPMI, supplemented with 10% FCS. The ACK buffer was removed by centrifugation and the splenocytes were re-suspended in 1 ml RPMI. The cells were counted using the Neubauer's chamber and 2 – 5 x 10^6 cells were taken for further analysis.

2.15.5 *In Vitro* Peptide Stimulation

Blood lymphocytes were obtained as described in chapter 2.15.4, on page 48. After two washing steps with FACS buffer, the cells were stimulated in a 96-well plate for 1 h at 37℃ with 1 µg/ml of the peptide, together with 0.25 µl of each CD28 and CD49d in a total volume of 100 µl RPMI (supplemented with 10% FCS). One hour later, Brefeldin A (10 µg/ml) was added and cells were incubated in the same conditions as above for additional 5 h. The cells were washed twice with FACS buffer and then used to stain against the surface marker.

2.15.6 Antibody Staining for Flow Cytometry

Surface markers on cells were stained according to the manufacture's protocol in a total volume of 50 µl FACS buffer for 30 min at 4℃ in darkness, followed by two washing steps with FACS buffer. The stained cells were either used for intracellular cytokine staining (ICCS) or direct acquisition in an Accuri, in an LSRII, or in an Fortessa apparatus in a total volume of 50 – 100 µl FACS buffer.

For ICCS, the cells were first stimulated with the desired peptides (see page 49, chapter "*In Vitro* Peptide Stimulation") and then stained for the desired surface markers. After the surface markers were stained with fluorescent antibodies, the cells were fixed with 100 µl IC Fixation buffer at 4℃ for 5 min, which was followed by 3 min permeabilisation with 100 µl permeabilisation buffer at 4℃. The cells were washed once with 200 µl permeabilisation buffer and incubated for 30 min at 4℃ with anti-IFN γ antibody (Ab) and anti-TNFα Ab in a total volume of 50 µl permeabilisation buffer. Afterwards, the cells were washed twice in permeabilisation buffer and re-suspended in 50 – 100 µl FACS buffer for acquisition. Peptide stimulated cells showed a better signal to noise ratio by IFNγ responses; thus, we focused on IFNγ as our readout system. Antigen-specific responses were discriminated from background noise by subtracting IFNγ responses in unstimulated controls from IFNγ values observed in peptide-stimulated samples. The cytometric results were analyzed with the FlowJo software.

2.15.7 IE3-Dextramer Staining for Flow Cytometry

The cells, derived from blood, were stained with the IE3-Dextramer prior to the surface marker staining. The peptide RALEYKNL, which is fused to the MHC proteins on the Dextramer backbone, was derived from the IE3 protein.

After cell isolation and lysis of the erythrocytes, the cells were washed two times with FACS buffer, prior to the staining with 3 µl of the IE3-Dextramer in a total volume of 30 µl FACS buffer for 15 min at RT in darkness. The surface marker staining with the desired antibodies was done according to the manufacturers' protocol in a total volume of 20 µl FACS buffer. These 20 µl were added on top of the 30 µl IE3-Detramer staining solution and incubated for additional 20 min at 4°C in darkness. The cells were washed two times with FACS buffer and re-suspended in 50 – 100 µl FACS buffer for acquisition in a flow cytometer within 2 h after staining.

2.15.8 Annexin V and 7AAD Staining

Splenocytes were harvested as described in chapter 2.15.4 on page 48. 4×10^6 splenocytes were used for the apoptosis staining. The cells were washed twice with PBS and incubated with a Live/Dead marker for 30 min at 4°C. The cells were washed with PBS and stained with CD8 – eFluor 450, CD62L – PE and CD44 – FITC for 15 min at 4°C. After an additional washing step with PBS, the cells were re-suspended in 250 µl binding buffer, before 5 µl Annexin V – APC and 2.5 µl 7AAD - PerCP were added and then incubated for 15 min at RT in the darkness. 250 µl binding buffer was added to the staining mix and the cells were acquired at a flow cytometer.

2.15.9 Counting Cell Populations in Absolute Terms

Cells were either counted in the Neubauer's chamber under a light microscope or with an Accuri flow cytometer. Therefore the cells were re-suspended in 100 µl FACS buffer from which 50 µl was acquired with an Accuri cytometer. For both methods the number of cells was calculated per ml.

2.15.10 Compensation with Single Stainings

For an accurate analysis of data, obtained by flow cytometry (FCM), the different fluorescent labels were mutually compensated. Therefore single-stained compensation beads (CompBeads) or splenocytes were used prior to each cell acquisition.

From a suspension, containing negative control CompBeads and anti-Rat and anti-Hamster CompBeads, 100 µl were added to as many wells in the 96-well plate as the number of antibodies in the staining panel, plus an extra well for the unstained control. Individual antibodies from the panel were added to the CompBeads and incubated for 30 min at 4℃ in darkness. After the incubation time, the CompBeads were washed twice with FACS buffer, re-suspended in 100 µl FACS buffer and analysed with a flow cytometer. To perform the compensation with Splenocytes, 10^6 cells were used per single staining.

2.16 Software and Statistical Analysis

Samples were acquired in LSRII and Fortessa cytometers fitted with the FACSDiva software (BD, version 6.1). For further analysis, Flowjo software (Tree Star, version 9.4) was used. Samples acquired at the Accuri cytometer were analysed by CFlow software (BD, version 1.0.227.4).

Statistical analyses were performed with GraphPad Prism software (GraphPad, version 5.04) and the Mann-Whitney U test (two-tailed) was used for comparisons between two groups.

3 Results

In this work, the influence of persistent gene expression during latency on the maintenance of MI was analysed. Therefore, a conditional knock-out of the MCMV genes *ie1/3*, which are known to induce MI, was performed *in vivo* using the Cre/*loxP* system. The genes *ie1/3* were flanked by *loxP* sequences and mice expressing ubiquitously an inducible Cre recombinase (Cre.ER^{T2}) were infected. The knock-out was induced at several months post infection (p.i.) by feeding mice with Tam. The effect of the knock-out on the MI was analysed by monitoring the accumulation of IE3-specific CD8$^+$ T cells throughout the experiment. However, unexpected results related to the toxic effect of Cre.ER^{T2} impaired the original plan. Thus the research goals were modified to investigate the effect of activated Cre.ER^{T2} on T_{EM} cells in more detail.

3.1 Generation of Recombinant MCMV: MCMV IE1/3^{flox}

The immediate-early genes *ie1* and *ie3* of MCMV were chosen to analyse the effect of persistent viral gene expression on the maintenance of MI. This was done because the mRNAs of *ie1* and *ie3* are detectable in latently infected organs several months after infection, hence in the absence of viral particles [72]. These genes encode peptides that induce MI in Balb/c and C57BL/6 mice [147, 148, 201]. To allow inducible deletion of *ie1/3* ORFs, the genomic region was flanked with *loxP* sequences using a traceless mutagenesis method, described in detail in chapter 2.13.2 [160], [202].

The intermediate and final BAC constructs of MCMV IE1/3^{flox} were confirmed by comparing their *EcoRV* restriction patterns to the MCMV WT BAC (Figure 12). The restriction pattern of the intermediate constructs carrying the *galK/Kn* cassette differed from the pattern of MCMV WT BAC in a manner that was consistent with *in silico* predictions. Constructs in which the *galK/Kn* cassettes were replaced by the *loxP*-sites, showed identical restriction patterns to MCMV WT BAC, because the *loxP* sequences are only 34 bp long and carry no restriction sites for *EcoRV*. The intermediate and final constructs were additionally validated by sequencing of the mutated sites. In all cases, no unwanted mutations could be identified.

To generate infectious virus, the MCMV IE1/3^{flox} DNA was transfected into primary MEFs from C57BL/6 mice. The SN was harvested, passaged five times to remove the BAC sequence and then used to prepare virus stocks [26]. The stability of the *loxP* sequences *in vitro* was confirmed by PCR of virus stock DNA at *loxP* insertion sites, followed by sequencing of PCR products (data not shown).

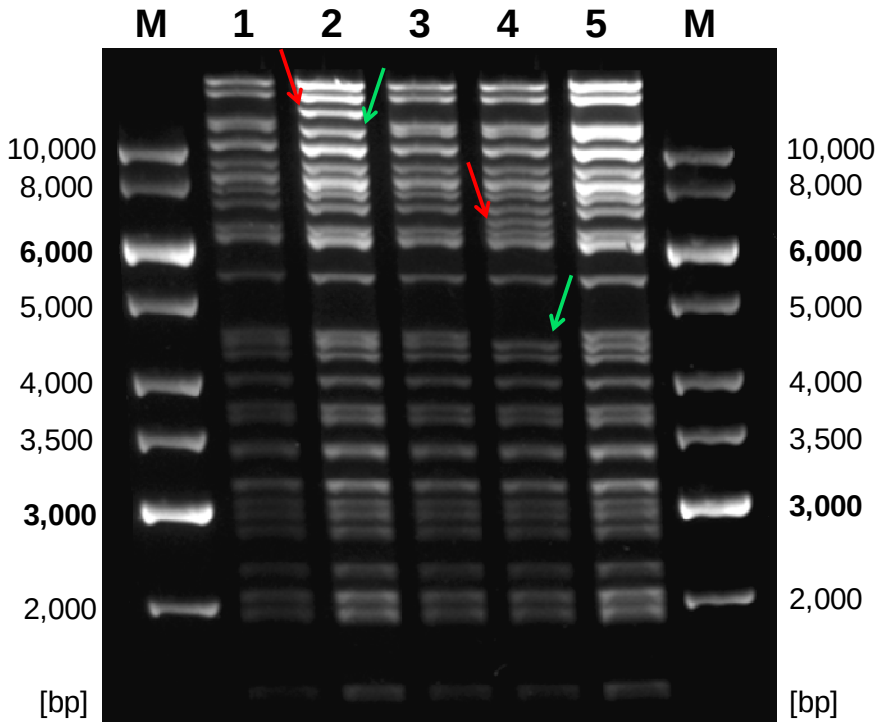

Figure 12 *EcoRV* restriction pattern analysis of the intermediate and final constructs of MCMV IE1/3^{flox} BAC.

The restriction analysis was performed on a horizontal agarose gel and bands bigger than 1,500 bp are displayed. Lanes were loaded with (M) the 1 kb DNA ladder, (1) the MCMV WT BAC, (2) the intermediate construct MCMV IE3 pA *galK/Kn* BAC, (3) the MCMV IE3 pA *loxP* BAC with one *loxP* site in the poly(A) tail, (4) the second intermediate construct MCMV IE3 pA *loxP* + IE1/3 Exon1 *galK/Kn* BAC and (5) the final MCMV IE1/3^{flox} BAC. The red arrows indicate additional bands in comparison to the MCMV WT BAC restriction pattern. The green arrows indicate absent bands in comparison to the MCMV WT BAC restriction pattern. Please note that only the intermediate constructs, with large *galK* and *Kn* gene insertions show different restriction patterns, while the more discrete modifications carrying only the *loxP* sites show no differences relative to the MCMV WT.

3.2 MCMV IE1/3^{flox} Replication *In Vitro* and *In Vivo*

To ensure that the fitness of the recombinant MCMV IE1/3^{flox} is similar to MCMV WT, the virus growth was examined by a multistep growth kinetic assay *in vitro* in NIH-3T3 cells. The cells were infected in three independent experiments with MCMV IE1/3^{flox} or MCMV WT at low MOI (0.1) and SNs were taken daily for 6 days and stored in -70°C until titration of infectious virus by plaque assay on MEFs could be performed. Results (Figure 13) argued that the recombinant virus replicated only slightly less well than the WT virus, but the final infectious titres were virtually identical.

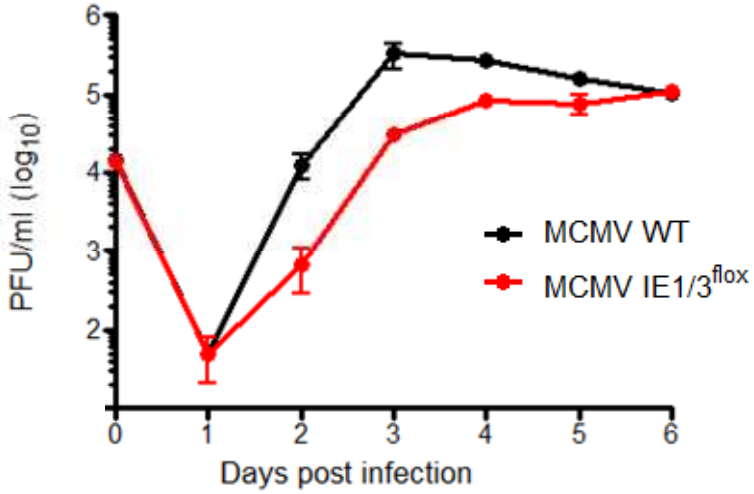

Figure 13 Insertion of *loxP* sequences into the *ie1/3* locus does not influence viral growth of MCMV IE1/3^{flox} *in vitro*.

NIH-3T3 cells were infected in three independent experiments with MOI 0.1 of MCMV IE1/3^{flox} or MCMV WT and SNs were harvested daily for 6 days and titrated on MEFs. Mean viral titres at indicated time points are shown. Vertical bars show the standard deviations (SD).

Recombinant viruses with normal growth *in vitro*, may nevertheless present growth deficits *in vivo* [129, 203-205]. Thus the viral replication was also tested in mice. C57BL/6 mice were i.p. infected with 10^6 PFU/mouse MCMV IE1/3^{flox} or MCMV WT. At five dpi, the spleen, liver and lungs were harvested in sterile conditions and stored at -70°C. Infectious virus titres were determined by p laque assay on MEFs. At dpi 21, infectious virus titres were determined in the salivary glands. As shown in Figure 14, the recombinant virus replicated comparably to the WT virus in all organs that have been tested.

In summary, the insertion of the *loxP* sequences in the *ie1/3* locus did not impair the viral growth *in vitro* or *in vivo*.

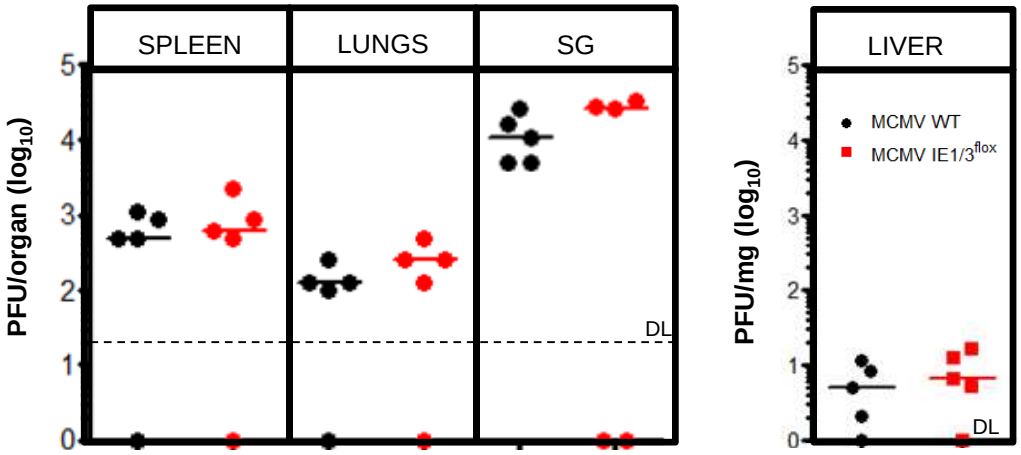

Figure 14 Insertion of *loxP* sequences into the *ie1/3* locus does not influence viral growth of MCMV IE1/3^{flox} *in vivo*.

In vivo growth of MCMV IE1/3^{flox} was compared to the growth of MCMV WT. C57BL/6 mice were i.p. infected with 10^6 PFU/mouse MCMV IE1/3^{flox} or MCMV WT. The spleen, liver and lungs were harvested under sterile conditions at five dpi, and the infectious virus titres were determined by plaque assay on MEFs. The infectious virus titres in the salivary glands were determined at 21 dpi. Each symbol represents one mouse and the horizontal lines indicate the medians.

3.3 Stability of Recombined MCMV IE1/3^{flox} *In Vivo*

The *in vivo* stability of the inserted *loxP* sequences was analysed by PCR of the *ie1/3* region after viral replication *in vivo*. C57BL/6 mice were i.p. infected with 10^6 PFU/mouse MCMV IE1/3^{flox} or MCMV WT and salivary glands were harvested 21 days later. The organ was homogenised and layered on MEFs expressing the inducible Cre.ER^{T2} (generated from transgenic C57BL/6 R26Cre.ER^{T2} mice), which were preincubated for 48 h with 1 µM Tam to induce the Cre recombinase. The cells were incubated at 37℃ for additional 48 h upon inf ection and the SNs were harvested and analysed by PCR with primers for the recombined *ie1/3* gene locus (Figure 15).

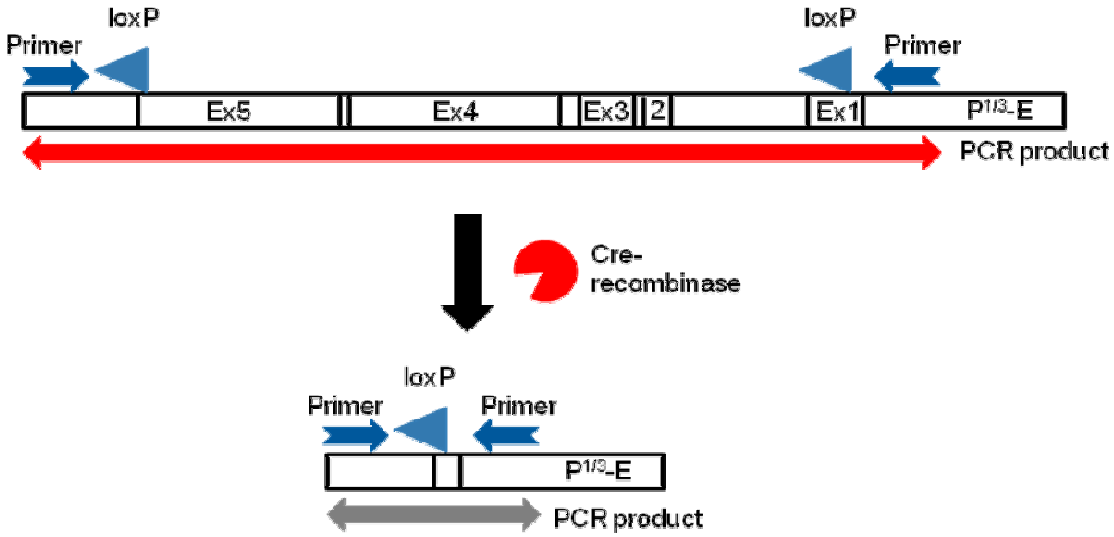

Figure 15 Schematic representation of the PCR products before and after the recombination of the *ie1/3* locus.

The Primer binding sites flank the ORF of *ie1/3*, including the *loxP* sequences. In absence of recombination, the PCR results in a large PCR product (red arrow), whereas upon recombination, the PCR results in a small PCR product (grey arrow).

The PCR product obtained from mice infected with MCMV IE1/3^{flox} was smaller than the MCMV WT samples and had the predicted size of 283 bp (Figure 16). Therefore, the *loxP* sequences remained stable during several replication cycles of the virus in its host and could be efficiently recombined.

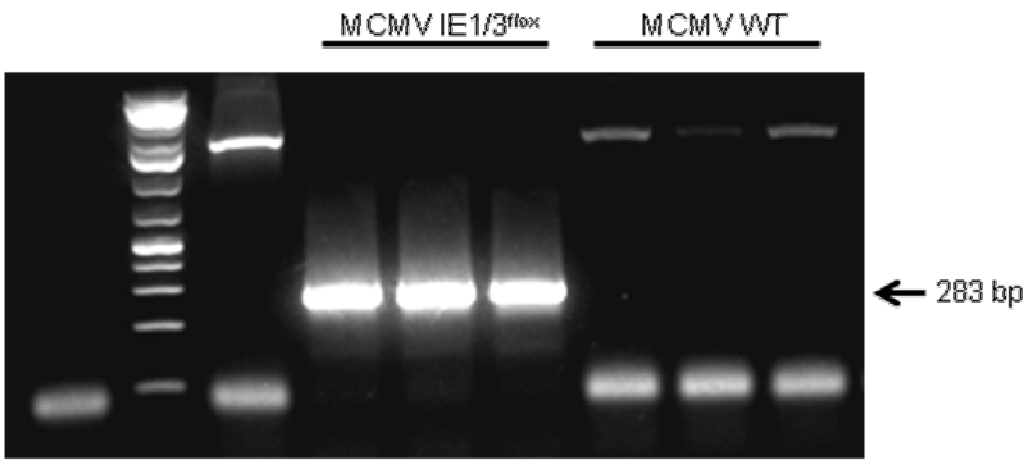

Figure 16 Function of the *loxP* sequences is stable after virus replication *in vivo*.

MEFs expressing inducible Cre.ER^{T2} were cultured for 48 h in presence of 1 µM Tam, then infected with the salivary gland homogenates from C57BL/6 mice, which were i.p. infected with 10^6 PFU/mouse MCMV IE1/3^{flox} or MCMV WT for 21 days, and cultured for additional 48 h. SNs were harvested and used to analyse the *ie1/3* gene locus for recombination by PCR with primers flanking the ORF of *ie1/3*. The PCR products were run on a horizontal agarose gel. The first lane shows the negative control, the second lane the 1kb DNA ladder and the third lane contains a PCR product with MCMV WT BAC as template. The 283 bp bands show PCR products consistent with Cre-mediated recombination of MCMV IE1/3^{flox}. The experiment was done with samples from three different mice for each virus.

3.4 MCMV IE1/3^{flox} Fails to Reactivate from IFNβ-Induced Latency *In Vitro*

The experimental strategy required that Cre recombinase targets a sufficient proportion of viral genomes to result in phenotypes that may be experimentally observed and quantified. Upon viral entry into the cell nucleus, cellular repressors and histones are recruited to the viral DNA (reviewed in [64]). It was shown that the MIEP is highly packaged in the latent phase of infection *in vivo* [74], and it was proposed that therefore transcription of immediate-early genes occurs only sporadically [47]. Therefore it was tested whether Cre recombinase may bind to and recombine a sufficient proportion of the introduced *loxP* sites to limit virus replication. Primary fibroblasts expressing constitutively active Cre recombinase under the strong CMV promoter were infected with MCMV. The growth and spread of the recombinant MCMV IE1/3^{flox} was not impaired at all (Figure 17), suggesting that during the lytical virus cycle, the MCMV gene expression and replication may be too rapid for the Cre-mediated targeting to recombine the *loxP* sites and thus inhibit MCMV replication and spread. Therefore, the efficient deployment of Cre recombinase required that viral genomes do not actively replicate, as it is the case during viral latency.

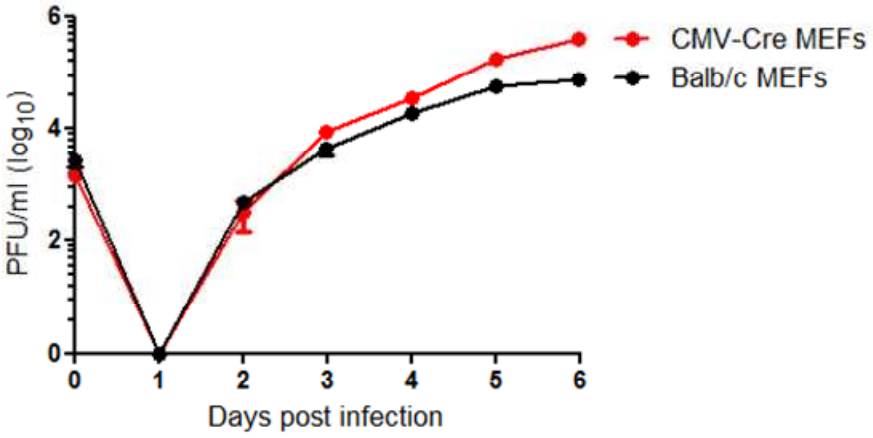

Figure 17 Cre recombinase does not influence viral growth of MCMV IE1/3^{flox} *in vitro*.

Cre-MEFs or Balb/c MEFs were infected in three independent experiments at MOI 0.1 of MCMV IE1/3^{flox} and SNs were harvested every day for 6 days and titrated on C57BL/6 MEFs. Mean viral titres at indicated time points are shown. Vertical bars show the standard deviations (SD).

Next, it was tested if the Cre recombinase may have a chance to recombine the latent genomes. A newly established *in vitro* latency model was used, where the addition of IFNβ reversibly inhibits immediate-early gene expression and MCMV replication in a manner consistent with viral latency (Dag *et al*, 2013, manuscript submitted). The addition of IFNβ one day before infection induces an anti-viral state in some, but not all, fibroblasts [206]. Hence, cells were infected with a very low MOI (0.0001), minimising the chance of infection of IFNß-unresponsive cells. The wells were scanned for cytopathic effects and MCMV IE1/3^{flox} replication occurred in almost all IFNβ-untreated wells, similar to MCMV WT (Figure 18). In cells which constantly received IFNβ, viral replication was blocked efficiently throughout the experiment. Removal of IFNβ at 7 dpi restored the replication of MCMV WT in some wells, whereas MCMV IE1/3^{flox} failed to reactivate in any well. These data indicated that Cre recombinase was able to act on the *loxP* sites and had sufficient time to excise the genes *ie1* and *ie3*, in the presence of IFNβ-recruited cellular repressors of viral transcription and replication.

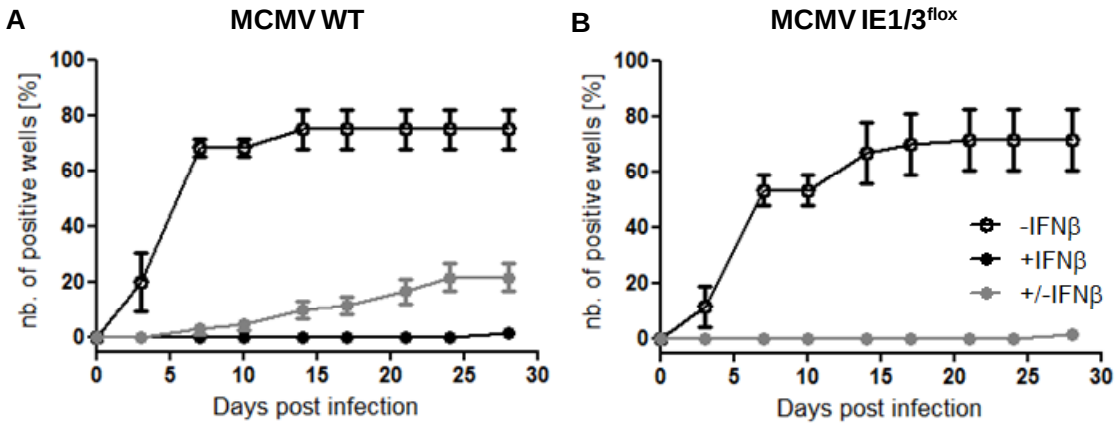

Figure 18 MCMV IE1/3^{flox} failed to replicate upon IFNβ removal from Cre-MEFs.

Cre-MEFs were treated with IFNβ and infected with MOI 0.0001 of MCMV WT (A) or MCMV IE1/3^{flox} (B). Control cells were infected in the absence of IFNβ (-IFNβ, open circle). At 7 dpi, IFNβ was removed in selected wells (+/-IFNβ, grey circle) or resupplied at regular interval (+IFNβ, black circle). At the indicated time points after infection, the mean percentage is listed and the SEM is shown in vertical bars from five independent experiments.

3.5 Immunological Phenotype of Recombinant MCMV IE1/3^{flox}

CD8$^+$ T cells specific for a peptide derived from the protein IE3 are known to accumulate and remain maintained at high levels during latent infection of C57BL/6 mice [147]. To test whether CD8$^+$ T cells are primed correctly with the peptide derived from the IE3 protein of the recombinant virus, mice were infected with 10^6 PFU/mouse MCMV IE1/3^{flox} or MCMV WT and the accumulation of IE3-specific CD8$^+$ T cells was monitored over several months. Transgenic C57BL/6 mice, expressing the inducible Cre recombinase Cre.ER^{T2} under the control of the ubiquitous Rosa26 promoter, were i.p. infected and monitored at 0, 7, 14, 28, 60, 90 and 120 dpi, when blood samples were analysed for IE3-specific CD8$^+$ T cells. The erythrocytes in the blood samples were lysed and the cells were incubated together with immunodominant peptides derived from the IE3, M45 or M38 gene. The cells were surface-stained with monoclonal antibodies against CD3, CD4, CD8a, CD44, and CD127 proteins and the response to the peptide stimulation was visualised by intracellular staining against IFNγ and TNFα (Table 1).

Table 1 Antibody panels used to analyse T cells from blood samples.

Antibody	Fluorochrom
anti-CD3	allophycocyanin-eFluor780
anti-CD4	Pacific Blue
anti-CD8a	PerCP-Cy5.5
anti-CD44	AlexaFluor700
anti-CD127	PE
anti–IFNγ	allophycocyanin
anti–TNFα	FITC

After staining, cells were acquired with the LSRII cytometer and their populations were analysed by progressive gating with FlowJo software. The exemplary blots shown in Figure 19 illustrate that the cells were gated on single T lymphocytes, positive for CD8a and IFNγ. Furthermore the $CD8^+$ T cells were separated for CD44 and CD127, to distinguish between T_{EM} cells ($CD44^+$, $CD127^-$), T_{CM} cells ($CD44^+$, $CD127^+$) and naive T cells ($CD44^-$, $CD127^+$). Fractions of populations were graphed and statistically analysed with Graphpad Prism software.

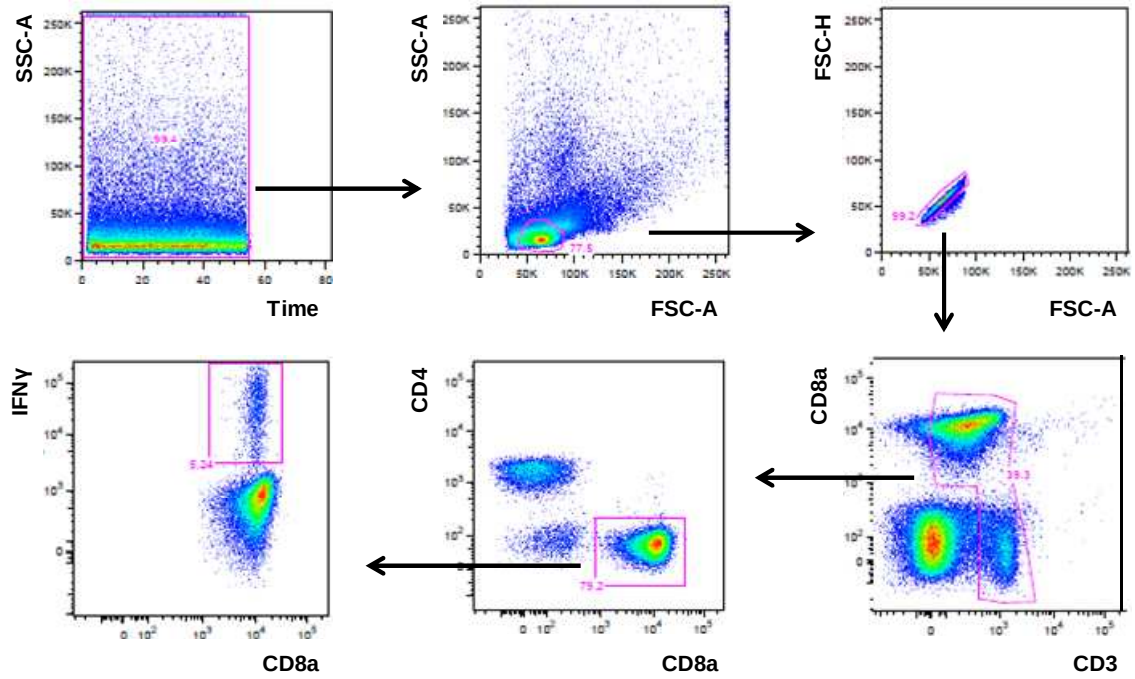

Figure 19 Gating strategy.

The cells were first gated on the time of acquisition to exclude artefacts from acquisition bursts towards the end of acquisition. Cells within the time gate were analysed for forward and side scatter area (FCS-A and SSC-A) to gate on the lymphocyte population. Cell doublets were excluded from the lymphocyte gate by gating on cells, whose FCS-A linearly correlated to FCS-H. These cells were then separated for CD3 expression, to focus on T cells and then separated with CD4 and CD8a, to identify the $CD8a^+$ T cells. The $CD8^+$ T cells were analysed for IFNγ expression.

To examine the response of the $CD8^+$ T cells to the peptide stimulation, the time kinetic of the relative numbers of the IFNγ positive $CD8^+$ T cells was measured (Figure 20). At 7 dpi, the numbers of $IFN\gamma^+$ $CD8^+$ T cells were comparable in mice infected with MCMV $IE1/3^{flox}$ and those infected with MCMV WT. At 14 and 28 dpi the $CD8^+$ T cells from mice infected with MCMV $IE1/3^{flox}$ showed a better response to the stimulation with IE3 peptide, but from 60 dpi on the responses were comparable again. The reason for the decreased response at the days 7 and 14 p.i. remained unclear, but it is unlikely a consequence of the experimental setup, because it happened before the Tam administration and the mice, infected with the recombinant virus did not show any deficit in IE3-specific $CD8^+$ T cell responses. The negative control, consisting of mice injected with PBS (MOCK group), showed no expansion of cells responding to the peptide.

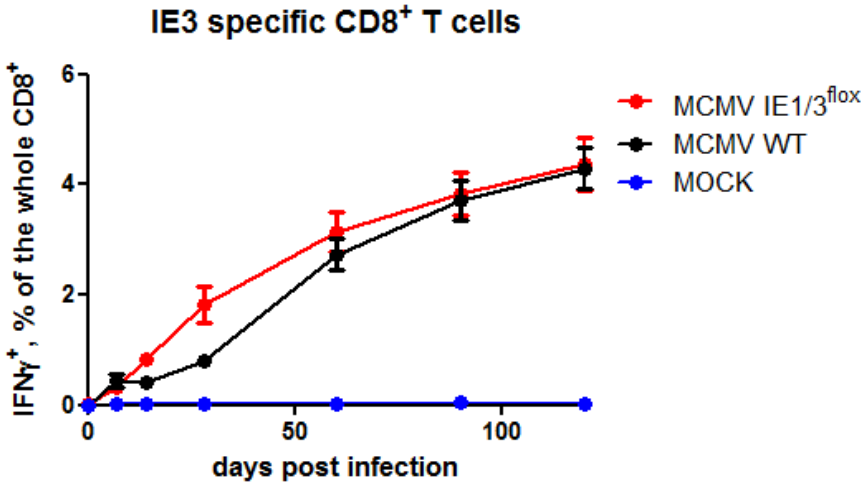

Figure 20 Kinetic of IE3-specific CD8$^+$ T cells is comparable between mice infected with MCMV IE1/3^{flox} and those infected with MCMV WT.

Mice (C57BL/6 R26Cre.ER^{T2}) were i.p. infected with 10^6 PFU of MCMV IE1/3^{flox} or MCMV WT. The MOCK group was injected with 200 µl PBS. Lymphocytes were stimulated with IE3 peptide and analysed by FCM. The groups reflect the IFNγ$^+$ cells in the CD3$^+$ CD4$^-$ CD8a$^+$ population at 0, 7, 14, 28, 60, 90 and 120 dpi. Pooled averages from 30 mice per group for infected mice and 15 mice for the MOCK group are shown. The error bars indicate the SEM.

To monitor the CD8$^+$ T cell immune response to MCMV IE1/3^{flox} in more detail, the kinetics of CD8$^+$ T cells specific for another inflationary peptide, M38, and one non-inflationary peptide, M45, were monitored and compared between the same infectious groups (Figure 21). The kinetics for both specific CD8$^+$ T cell responses showed no difference.

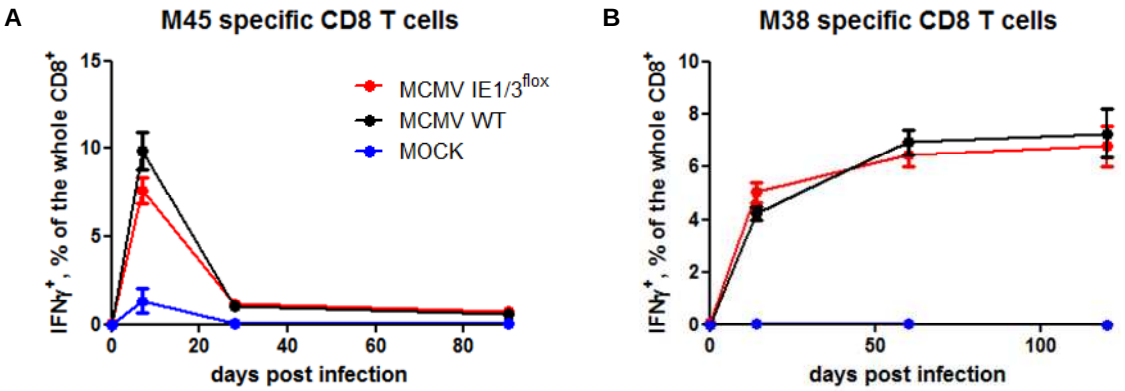

Figure 21 Kinetics of M45- and M38-specific CD8$^+$ T cells are comparable between mice infected with MCMV IE1/3^{flox} and those infected with MCMV WT.

Mice (C57BL/6 R26Cre.ER^{T2}) were i.p. infected with 10^6 PFU MCMV IE1/3^{flox} or MCMV WT. The MOCK group was injected with 200 µl PBS. Lymphocytes were stimulated with M45 or M38 peptide and analysed by FCM. The groups reflect the IFNγ$^+$ cells out of the CD3$^+$ CD4$^-$ CD8a$^+$ population at 0, 7, 28 and 90 dpi for stimulation with M45 peptide **(A)** and 0, 14, 60 and 120 dpi for stimulation with M38 peptide **(B)**. Pooled averages from 30 mice per group for infected mice and 15 mice for the MOCK group are shown. The error bars indicate the SEM.

Taken together, the kinetics of CD8$^+$ T cell responses indicated that the IE3 peptide is detected by CD8$^+$ T cells upon MCMV IE1/3^{flox} or MCMV WT infection, and that the responses to other viral peptides were also not impaired. Moreover, the results argued that non-activated Cre recombinase Cre.ER^{T2} does not abrogate the accumulation of the IE3-specific CD8$^+$ T cells in the mice infected with the recombinant virus.

3.6 Conditional Knock-Out of IE1/3 *In Vivo*

The data argued that the recombinant virus MCMV IE1/3^{flox} may be able to establish MI in the C57BL/6 R26Cre.ER^{T2} mice, because IE3-specific CD8$^+$ T cells accumulated with the same frequencies as the MCMV WT control. We reasoned that the knock-out of the *ie1/3* genes may be induced once the virus is latent by administering Tam to the mice. Tam administration was initiated at four months p.i. (120 dpi), because the infectious viral particles are not detectable anymore, but viral genomes are still present in the organs at this time [52], [207].

Latency also means that transcriptional repressors are bound to the MIEP and other viral promoters [74] to block viral gene expression. In the latent phase of infection the amount of deacetylated histons bound to the viral genome is greatly increased. Especially the MIEP is highly packed at >120 dpi [78]. However, random asynchronous transcription from the MIEP was observed during viral latency [71]. Therefore, it was

assumed that DNA may be exposed to the Cre recombinase during the transcriptional events that transiently relax the packaging of the viral genome. It followed that Tam would have to be administered for an extended time (4 weeks) to allow sufficient time for the Cre recombinase to bind to the *loxP* sites in the MIEP region once the DNA is not blocked by cellular repressors. To avoid prolonged stress to mice, Tam was administered in food pellets. It has been shown that the administration of pellets containing 360 mg/kg Tam for a period of 4 weeks was sufficient to ensure thorough recombination of chromosomally localised *loxP* target [184]. Pellets containing 800 mg/kg or 400 mg/kg of Tam were tested for toxicity, and pellets with 800 mg/kg showed severe toxicity. On the other hand, no mortality was observed in uninfected female or male R26Cre.ER^{T2} mice upon administration of 400 mg/kg (data not shown). Hence, pellets containing 400 mg/kg of Tam were used in these experiments.

15 mice infected with the MCMV IE1/3^{flox} or MCMV WT were fed for 4 weeks with Tam pellets, while 15 control mice from each group were maintained on standard diet. 5 out of 30 mice fed with Tam died during the experiment. The mechanism of the toxicity and death has not been studied, and it remains unclear if it is related to direct Tam toxicity or caused by the activation of the Cre recombinase, but interestingly, all 5 mice which died were females.

After four weeks of Tam administration, the mice were bled again and the lymphocytes were stimulated with the IE3 peptide. Mice fed with Tam showed a reduction of around 40% of the IE3-specific CD8$^+$ T cells (Figure 22). Surprisingly, this drop was not restricted to the group infected with MCMV IE1/3^{flox}, because it was observed to the same extent in the group infected with MCMV WT.

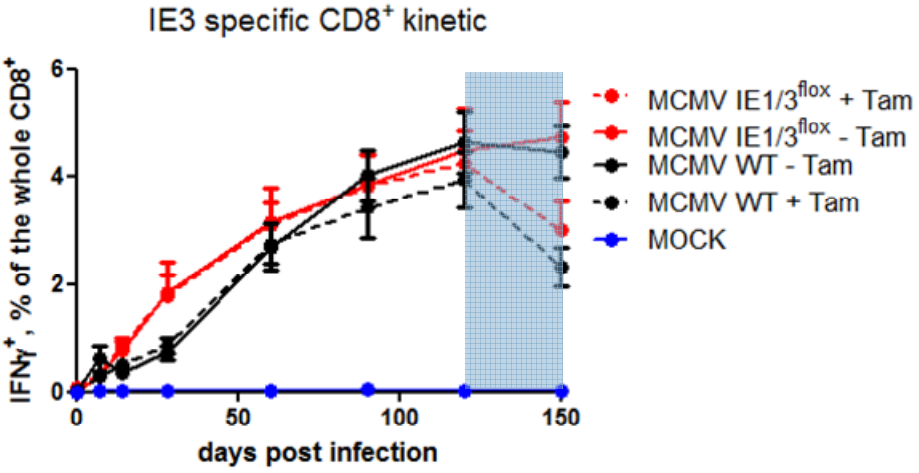

Figure 22 Decreased percentage of IE3-specific $CD8^+$ T cells in R26Cre.ER^{T2} mice infected with MCMV $IE1/3^{flox}$ and those infected with MCMV WT upon Tamoxifen administration.

Mice (C57BL/6 R26Cre.ER^{T2}) were i.p. infected with 10^6 PFU MCMV $IE1/3^{flox}$ or MCMV WT. The MOCK group was injected with 200 µl PBS. The lymphocytes were stimulated with IE3 peptide and analysed by FCM for IFNγ production in cells out of the $CD3^+$ $CD4^-$ $CD8a^+$ population at 0, 7, 14, 28, 60, 90, 120 and 150 dpi. Mice were fed with Tam from 120 to 150 dpi (shaded rectangular area). Group averages from 15 mice per group are shown. The error bars indicate the SEM.

To define whether the loss of cells affected only the MCMV-specific T cells, three major $CD8^+$ T cell subsets were analysed, namely the naive T cells ($CD8^+$ $CD44^-$ $CD127^+$), the $CD8^+$ T_{CM} cells ($CD8^+$ $CD44^+$ $CD127^+$) and the $CD8^+$ T_{EM} cells ($CD8^+$ $CD44^+$ $CD127^-$). As shown in Figure 23, the percentage of T_{EM} cells decreased in all mice exposed to Tam. On the other hand, the fraction of cells in the T_{CM} compartment increased, whereas the percentage of naive T cells was not affected.

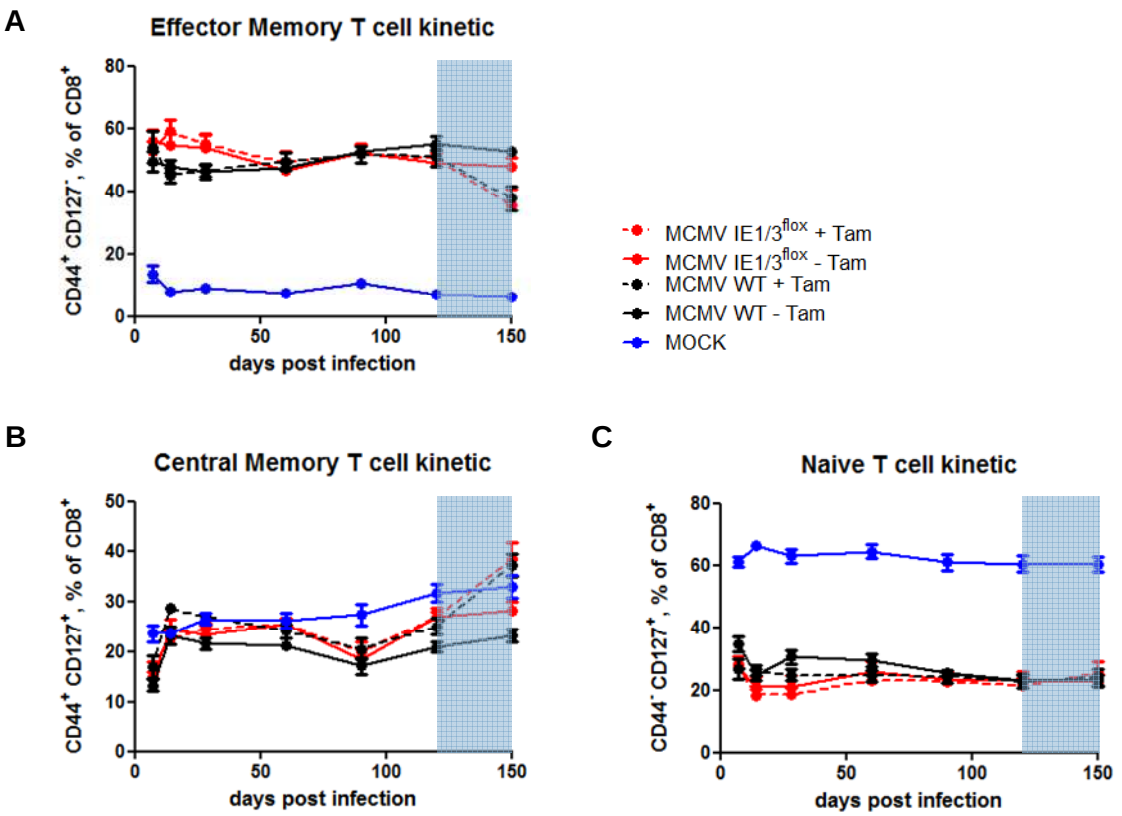

Figure 23 Decreased percentage of effector memory CD8$^+$ T cells in R26Cre.ER^{T2} mice infected with MCMV IE1/3^{flox} and those infected with MCMV WT upon Tamoxifen administration.

Mice (C57BL/6 R26Cre.ER^{T2}) were i.p. infected with 10^6 PFU MCMV IE1/3^{flox} or MCMV WT. The MOCK group was injected with 200 µl PBS. Mice were fed with Tam from 120 to 150 dpi (shaded rectangular area). The lymphocytes were analysed by FCM. Shown are the kinetics of three subsets of the CD3$^+$ CD4$^-$ CD8a$^+$ population at 0, 7, 14, 28, 60, 90, 120 and 150 dpi. (**A**) The T_{EM} cell compartment was defined as CD44$^+$ and CD127$^-$. (**B**) The T_{CM} cell compartment was defined as CD44$^+$ and CD127$^+$. (**C**) The naive T cell compartment was defined as CD44$^-$ and CD127$^+$. All panels show group averages from 15 mice per group. The error bars indicate the SEM.

To define if the effect of Tam treatment on the CD8$^+$ T cells is reversible, Tam treated mice were reverted to the standard diet and the T cells were monitored for two more months. As seen in Figure 24, the percentage of IE3-specific CD8$^+$ T cells increased back to levels comparable to the groups which were not fed with Tam. Moreover, the relative numbers of T_{EM} cells and T_{CM} cells returned to the levels seen in the control groups (Figure 25). Therefore, the transient loss of virus-specific CD8$^+$ T cells was not consistent with a permanent deletion of the *ie1/3* gene from the virus genome.

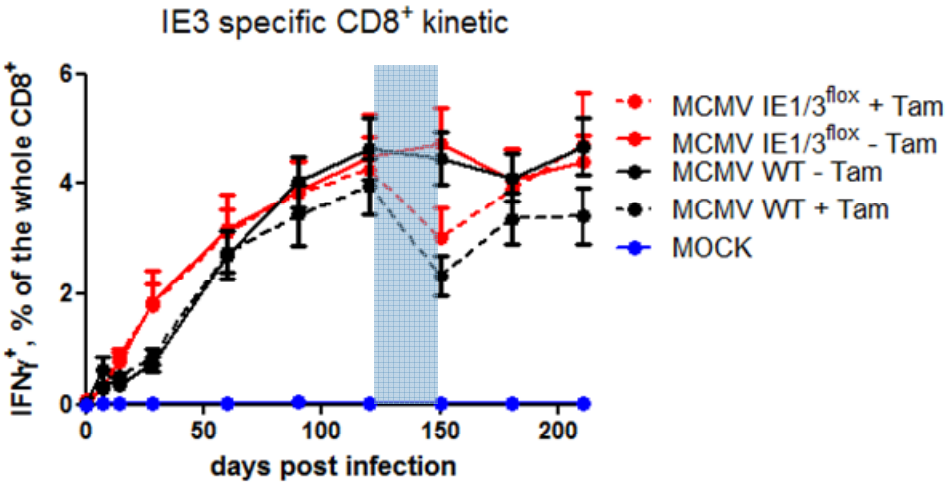

Figure 24 Reversible decrease of IE3-specific CD8$^+$ T cells upon Tamoxifen administration.

Mice (C57BL/6 R26Cre.ER^{T2}) were i.p. infected with 10^6 PFU MCMV IE1/3^{flox} or MCMV WT. The MOCK group was injected with 200 µl PBS. Mice were fed with Tam from 120 to 150 dpi (shaded rectangular area). The lymphocytes were stimulated with IE3 peptide and analysed by FCM. Data reflect the percentage of IFNγ$^+$ cells out of the CD3$^+$ CD4$^-$ CD8a$^+$ population at 0, 7, 14, 28, 60, 90, 120, 150, 180 and 210 dpi. Averages from 15 mice per group are shown. The error bars indicate the SEM.

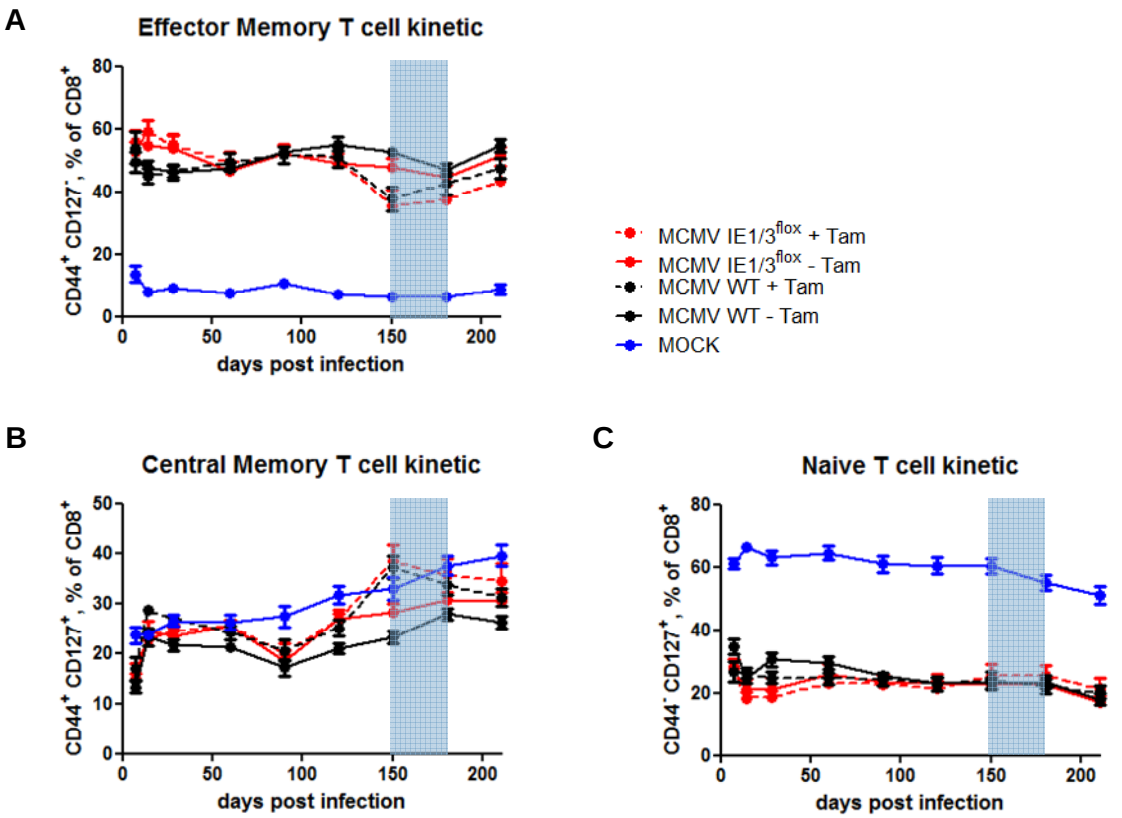

Figure 25 Reversible decrease of effector memory T cells upon Tamoxifen administration.

Mice (C57BL/6 R26Cre.ER^{T2}) were i.p. infected with 10^6 PFU MCMV IE1/3^{flox} or MCMV WT. The MOCK group was injected with 200 µl PBS. Mice were fed with Tam from 120 to 150 dpi (shaded rectangular area). The lymphocytes were analysed by FCM. Data reflect the percentage of cells in the three subsets of the CD3$^+$ CD4$^-$ CD8a$^+$ population at 0, 7, 14, 28, 60, 90, 120, 150, 180 and 210 dpi. (**A**) The T_{EM} cell compartment was defined as CD44$^+$ and CD127$^-$. (**B**) The T_{CM} cell compartment was defined as CD44$^+$ and CD127$^+$. (**C**) The naive T cell compartment was defined as CD44$^-$ and CD127$^+$. All panels show averages from 15 mice per group. The error bars indicate the SEM.

It remained unclear if the effects of Tam were due to its toxicity (direct, or mediated by an activated Cre.ER^{T2}), or if they reflected a transient transcriptional suppression of MCMV genes, and consequently for induction of CD8$^+$ T cells by the viral genome.

3.7 Influence of Tamoxifen on MCMV-Specific T Cells

While the administration of Tam had a negative effect on the T_{EM} cell compartment of MCMV-infected R26Cre.ER^{T2} mice, it remained unclear if Tam would have the same effect on T cells of mice that do not express the Cre.ER^{T2}.

C57BL/6 WT mice were infected with 10^6 PFU MCMV WT and fed with pellets containing 400 mg/kg Tam from 60 to 90 dpi, once the IE3-specific CD8$^+$ T cells reached a level of more than 4% of the CD8$^+$ T cell pool. The relative counts of different CD8$^+$ T cell subsets were compared between the groups before the infection, to confirm that the increase in IE3-specific cells was due to virus infection, and before and after the Tam administration (at 60 and 90 dpi, respectively), to monitor the gain or the loss of virus-specific cells relative to the count prior to the administration. As shown in Figure 26, the percentage of IE3-specific CD8$^+$ T cells did not decrease upon Tam treatment. In fact, consistent with data from R26Cre.ER^{T2} mice (Figure 20), the fraction of IE3-specific CD8$^+$ T cells increased between days 60 and 90, and this gain was not slowed down by Tam administration.

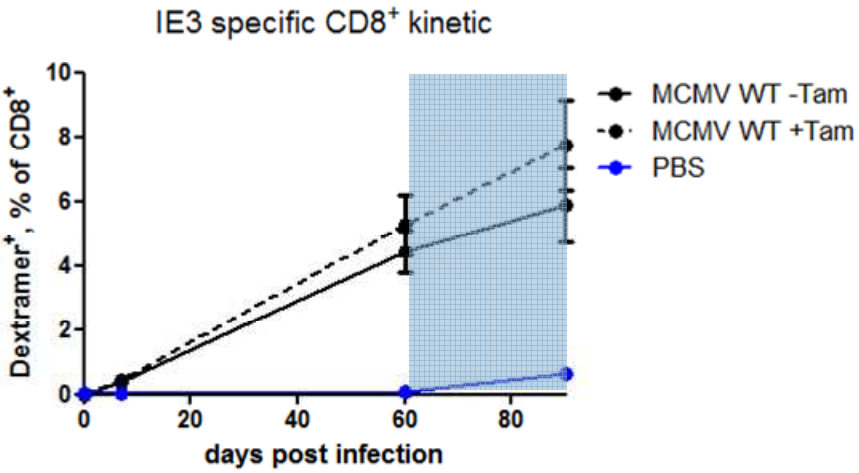

Figure 26 Administration of Tamoxifen to C57BL/6 mice, infected with MCMV WT, had no effect on the relative numbers of IE3-specific CD8$^+$ T cells.

Mice (C57BL/6) were i.p. infected with 10^6 PFU MCMV WT. The MOCK group was injected with 200 µl PBS. Mice were fed with Tam from 60 to 90 dpi (shaded rectangular area). The lymphocytes were stained with an IE3-specific pMHC Dextramer and analysed by FCM. Data represent the Dextramer$^+$ cells out of the CD3$^+$ CD4$^-$ CD8a$^+$ population at 0, 7, 60 and 90 dpi. Averages percentages from 15 mice per group are shown. The error bars indicate the SEM.

The $CD8^+$ T cells were further analysed for the expression of the surface marker CD44 and CD62L. In all three T cell compartments, T_{EM} cells ($CD8^+$ $CD44^+$ $CD62L^-$), T_{CM} cells ($CD8^+$ $CD44^+$ $CD62L^+$) and naive T cells ($CD8^+$ $CD44^-$ $CD62L^+$), there was no difference between the MCMV-infected groups fed with Tam pellets or with standard mouse chow (Figure 27). In the memory T cell subset a minor difference at 90 dpi could be observed, which did not reach to levels of statistical significance.

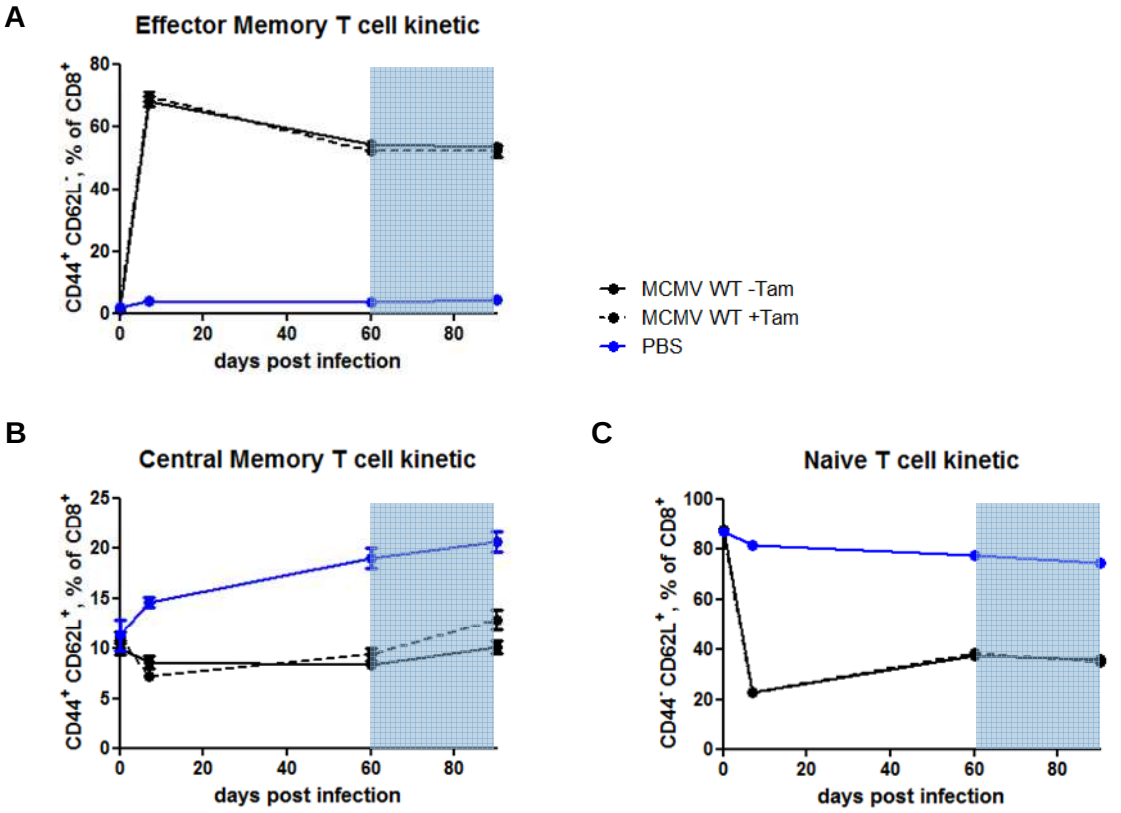

Figure 27 Kinetic of effector memory $CD8^+$ T cells is not influenced by administration of Tamoxifen to C57BL/6 mice, infected with MCMV WT.

Mice (C57BL/6) were i.p. infected with 10^6 PFU MCMV WT. The MOCK group was injected with 200 µl PBS. Mice were fed with Tam from 60 to 90 dpi (shaded rectangular area). The lymphocytes were analysed by FCM. Data represent the fraction of indicated T cell subsets in the $CD3^+$ $CD4^-$ $CD8a^+$ population at 0, 7, 60 and 90 dpi. (**A**) The T_{EM} cell compartment was defined as $CD44^+$ and $CD62L^-$. (**B**) The T_{CM} cell compartment was defined as $CD44^+$ and $CD62L^+$. (**C**) The naive T cell compartment was defined as $CD44^-$ and $CD62L^+$. All panels show percentage averages from 15 mice per group. The error bars indicate the SEM.

To exclude the possibility that Tam has generalised toxic effects on the entire $CD8^+$ T cell compartment, reducing the total $CD8^+$ T cell count, while leaving the fraction of each subset intact, the absolute values have been investigated. At 90 dpi, the blood lymphocytes were stained with anti-CD8a, anti-CD44, anti-62L and anti-CD127 and

counted with an Accuri cytometer. The graphs in Figure 28 show the $CD8^+$ T cell compartments phenotyped according to CD44 and CD62L expression. No significant difference between the infected groups could be seen. Again, in the T_{CM} cell compartment there was a small difference between the group treated with Tam and the group without Tam, but the difference was not statistically significant. These data indicated that Tam does not influence the relative and absolute numbers of $CD8^+$ T cells in MCMV-infected mice which do not express the $Cre.ER^{T2}$.

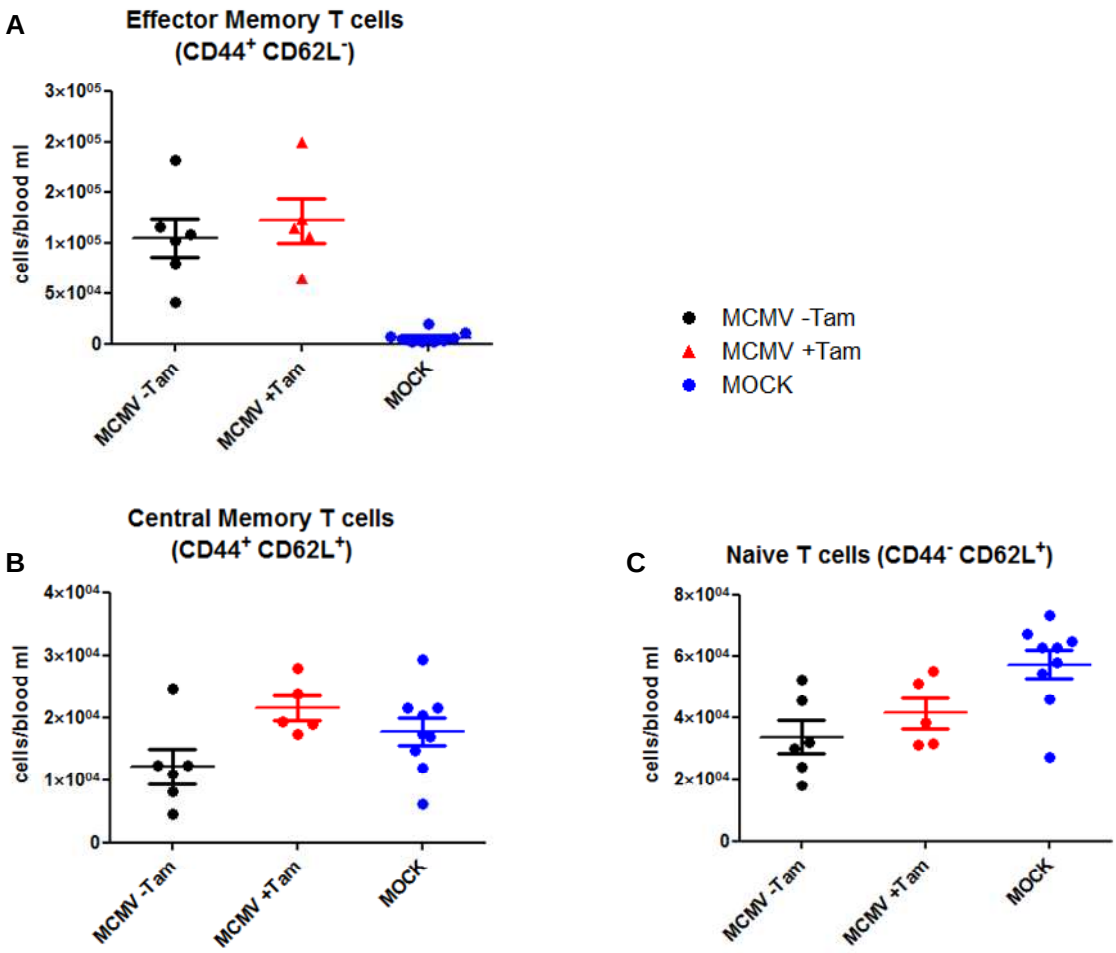

Figure 28 Absolute numbers of effector memory $CD8^+$ T cells are not influenced by administration of Tamoxifen to C57BL/6 mice, infected with MCMV WT.

Mice (C57BL/6) were i.p. infected with 10^6 PFU MCMV WT. The MOCK group was injected with 200 µl PBS. Mice were fed with Tam from 60 to 90 dpi. The lymphocytes were stained with anti-CD8a, anti-CD44, anti-CD62L and anti-CD127 and analysed by FCM at 90 dpi. (**A**) The T_{EM} cell compartment was defined as $CD44^+$ and $CD62L^-$. (**B**) The T_{CM} cell compartment was defined as $CD44^+$ and $CD62L^+$. (**C**) The naive T cell compartment was defined as $CD44^-$ and $CD62L^+$. All panels show percentage averages from up to 9 mice per group. The error bars indicate the SEM.

3.8 Effects of Tamoxifen Treatment in MCMV-Infected R26Cre.ER^{T2} Mice on Different Peptide-Specific CD8$^+$ T Cells

Tam transiently decreased the percentage of MCMV-specific T cells and the relative size of the T_{EM} cell compartment in R26Cre.ER^{T2} mice, but not in the parental C57BL/6 strain.

In the previous chapters, the CD8$^+$ T cells specific for the inflationary peptide IE3 were analysed, which predominantly exhibit a T_{EM} phenotype. To define whether the loss of cells affected also the non-inflationary CD8$^+$ T cells, the M45-specific T cells were analysed, which mostly have a T_{CM} phenotype. As shown in Figure 29, the percentage of M45-specific CD8$^+$ T cells was not affected upon the administration of Tam.

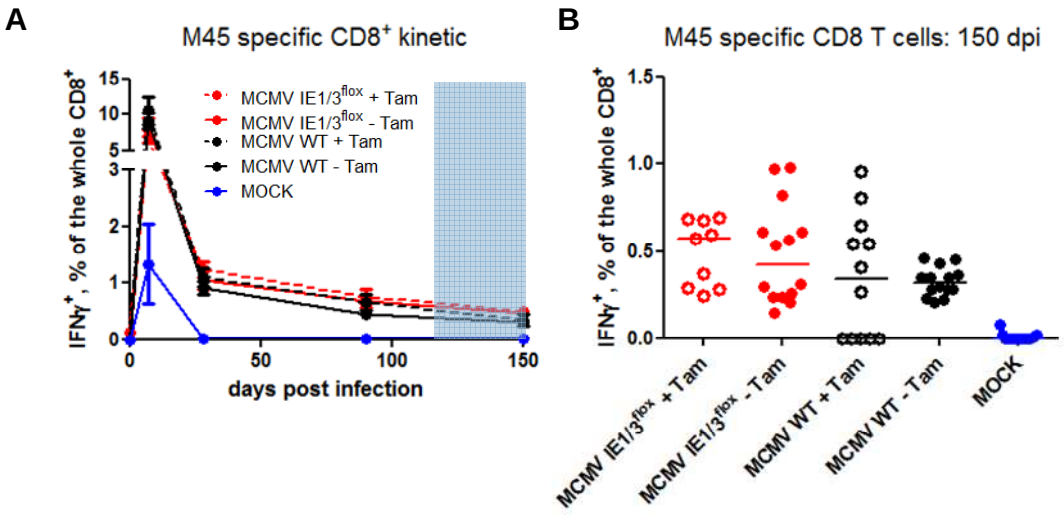

Figure 29 Administration of Tamoxifen to MCMV-infected R26Cre.ER^{T2} mice did not affect the percentage of M45-specific CD8$^+$ T cells.

Mice (C57BL/6 R26Cre.ER^{T2}) were i.p. infected with 10^6 PFU MCMV IE1/3^{flox} or MCMV WT. The MOCK group was injected with 200 µl PBS. The lymphocytes were stimulated with M45 peptide and analysed by FCM for IFNγ production in cells out of the CD3$^+$ CD4$^-$ CD8a$^+$ population. Mice were fed with Tam from 120 to 150 dpi (shaded rectangular area). (**A**) IFNγ$^+$ cells from 0, 7, 28, 90 and 150 dpi are shown. Group averages from up to 15 mice per group are shown. The error bars indicate the SEM. (**B**) IFNγ$^+$ cells out of the CD3$^+$ CD4$^-$ CD8a$^+$ population from 150 dpi are shown. Each symbol represents one mouse and the horizontal lines indicate the medians.

For historical reasons, $Rag2^{flox/flox}$xR26Cre.ER^{T2} mice were infected with a recombinant MCMV in an independent experiment. In this mouse strain, the Cre.ER^{T2} is expressed under the control of the Rosa26 promoter and both alleles of the *rag2* are floxed. In theory, the somatic recombination would be prevented in the lymphocyte precursor cells of these mice upon Tam administration, and as a result naive T cells should not be generated anymore. Therefore, this study was initiated to investigate whether the recruitment of naive T cells to the IE3-specific T cells might have a significant influence on their accumulation. Due to the effects of Tam on the $CD8^+$ T cell compartment in R26Cre.ER^{T2} mice (see chapter 3.6), the outcomes of this study could not be used to investigate the original question. However, this experiment was performed in a similar setup as the experiments shown in chapters 3.6 and 3.7, especially concerning the route of infection, the establishment of latent MCMV infection, as well as the Tam administration. Therefore, the results of this study were used to analyse whether the Tam induced effect was specific for the IE3-specific T cells within the T_{EM} cell pool or if it might influence also other inflationary T cells in mice expressing Cre.ER^{T2}.
The virus, used in this experiment expresses the immunodominant peptide SSIEFARL in the IE2 locus (MCMV $IE2^{SL}$). SSIEFARL is a peptide derived from the envelope protein gB of Herpes Simplex Virus 1 (HSV-1) and the recombinant virus has been recently described and analysed in our group [155]. The T cells specific for the SSIEFARL peptide inflate during latency and the majority of these cells belong to the T_{EM} cell subset.

The mice were infected with 10^6 PFU of the recombinant MCMV. The $CD8^+$ T cells were monitored throughout the experiment and analysed by FCM. The mice were fed with pellets containing 400 mg/kg Tam from 60 to 90 dpi. After the administration of Tam, the relative counts of different $CD8^+$ T cell subsets and peptide-stimulated T cells were analysed. As shown in Figure 30 the Tam administration led to a reversible drop of SSIEFARL and M38-specific T cells.

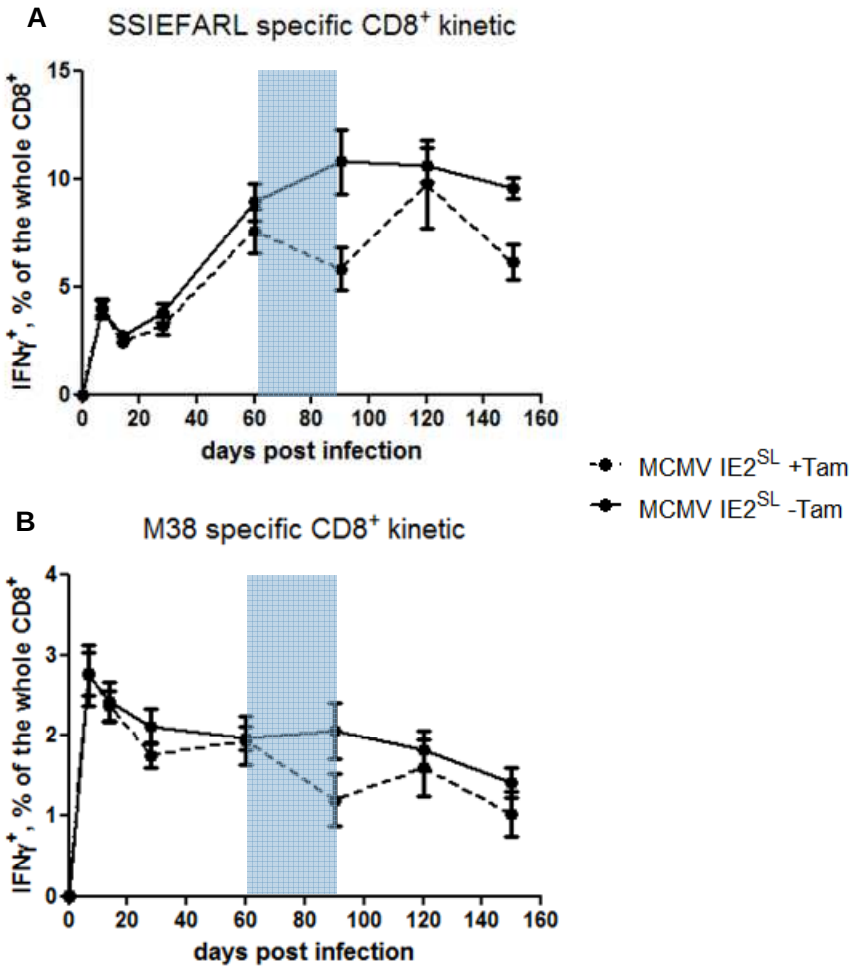

Figure 30 Administration of Tamoxifen leads to a reversible drop in the percentage of MCMV-specific CD8$^+$ T cells.

Mice (C57BL/6 Rag2$^{flox/flox}$xR26Cre.ER^{T2}) were infected i.p. with 10^6 PFU MCMV IE2^{SL}. Mice were fed with Tam from 60 to 90 dpi (shaded rectangular area). The lymphocytes were stimulated with peptide and analysed by FCM. The groups reflect the IFNγ$^+$ cells out of the CD3$^+$ CD4$^-$ CD8a$^+$ population at 0, 7, 14, 28, 60, 90, 120 and 150 dpi for stimulation with **(A)** SSIEFARL peptide or **(B)** M38 peptide. Averages from 15 mice per group are shown. The error bars indicate the SEM.

The CD8$^+$ T cells were also analysed for the percentage of effector memory (CD44$^+$ CD127$^-$), central memory (CD44$^+$ CD127$^+$) and naive (CD44$^-$ CD127$^+$) T cells, to define if these T cell subsets would be influenced by Tam administration. The same results as in chapter 3.6 could be observed. The percentage of T_{EM} cells dropped upon Tam treatment and the percentage of memory T cells increased at the same time, in a reversible manner. 150 dpi the percentages of effector memory and memory T cells were similar to those of untreated mice.

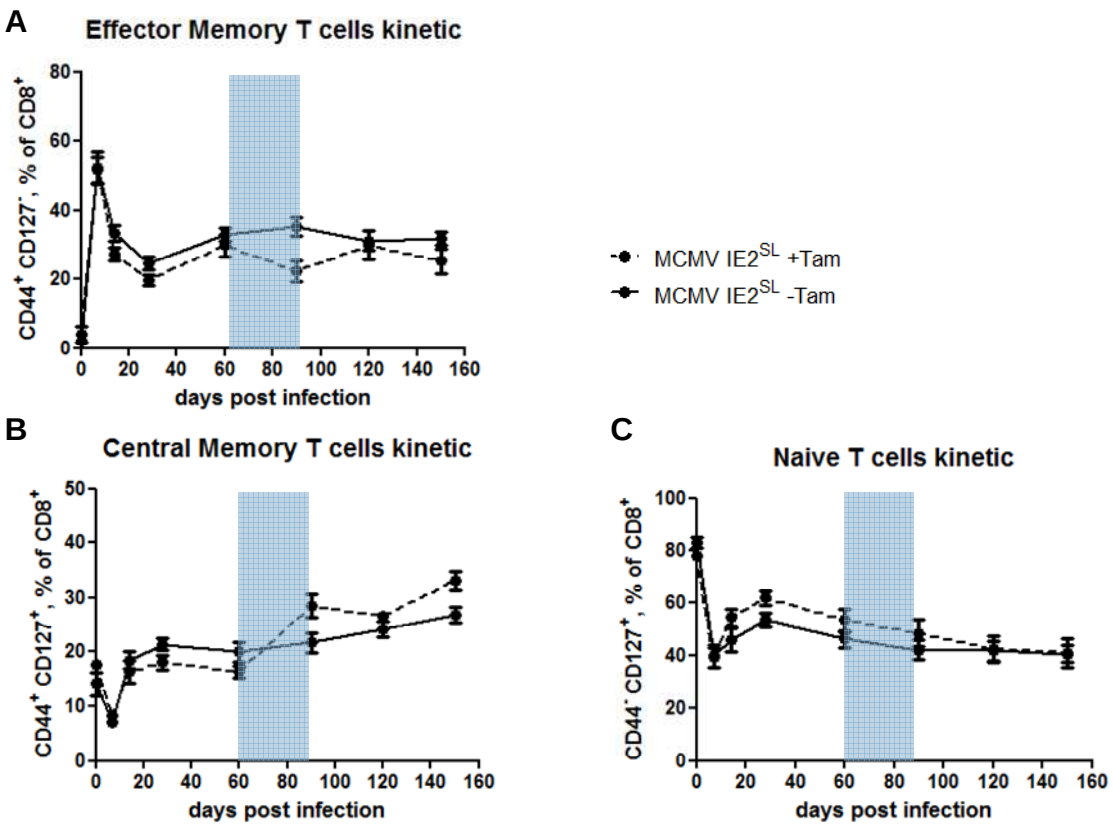

Figure 31 Reversible effect of activated Cre.ER^{T2} on effector memory CD8$^+$ T cells.

Mice (C57BL/6 Rag2$^{flox/flox}$xR26Cre.ER^{T2}) were infected i.p. with 10^6 PFU MCMV IE2^{SL}. Mice were fed with Tam from 60 to 90 dpi (shaded rectangular area). The lymphocytes were analysed by FCM. Data reflect the percentages of cells in the three subsets of the CD3$^+$ CD4$^-$ CD8a$^+$ population at 0, 7, 14, 28, 60, 90, 120 and 150 dpi. (**A**) The T_{EM} cell compartment was defined as CD44$^+$ and CD127$^-$. (**B**) The T_{CM} cell compartment was defined as CD44$^+$ and CD127$^+$. (**C**) The naive T cell compartment was defined as CD44$^-$ and CD127$^+$. All panels show averages from 15 mice per group. The error bars indicate the SEM.

These data suggested that the Tam induced effect in R26Cre.ER^{T2} mice is not restricted to IE3-specific T cells but may influence all MCMV-specific inflationary T cells.

Furthermore, this effect was reversible upon Tam retraction, but it remained somewhat unclear from which T cell compartment the T_{EM} cell pool was refilled. Theoretically, in MCMV-infected Rag2$^{flox/flox}$xR26Cre.ER^{T2} mice, the percentage of T_{EM} cells should not come back upon Tam administration as it occurred in this study, if the recruitment of newly generated naive T cells to that pool plays an important role. Therefore, although the absolute count of naive T cells was not verified upon Tam treatment to analyse the successful recombination of the *Rag2* gene, these results would corroborate the previously published data that recent thymic emigrants are not required for the maintenance of T_{EM} cells in a MCMV infection [158].

3.9 Toxic Effect of Activated Cre.ER^{T2} on the Effector Memory T Cell Subset

Activated Cre.ER^{T2} has a toxic effect on immature cells of the hematopoietic cell lineage [208]. It was described that the conditional Cre recombinase has access to the temporally unpacked genome in highly proliferating cells and is able to induce chromosomal abnormalities in them [208]. In this line, Thyagarajan *et al.*, could show that the mammalian genome contains active recombinase recognition sites, the so-called pseudo-*lox* sites, which support Cre-mediated chromosomal integration and excision [209].

A possible explanation for the decrease in the relative counts of T_{EM} cells was that these cells still proliferated and thus suffered from Cre-induced chromosomal abnormalities. To investigate this scenario, the different T cell subsets were analysed for apoptosis upon Tam treatment of latently infected R26Cre.ER^{T2} mice.

C57BL/6 R26Cre.ER^{T2} mice infected for 21 month with 10^6 PFU/mouse MCMV WT were fed with pellets containing 400 mg/kg Tam for 2 weeks. Upon Tam treatment, the splenic lymphocytes were harvested and stained with Annexin V antibodies and 7AAD. A conjugate of Annexin V and a fluorescent label can be used to detect apoptotic cells, as Annexin V binds to phosphatidylserine which is exposed on the cell surface only during apoptosis. 7AAD is a fluorescent chemical, intercalating into double-stranded DNA, but unable to pass the intact cell membrane. Therefore it is used to detect apoptotic or necrotic cells, in which the cell membrane is already permeable. Using these compounds in combination allows dividing the apoptotic cells into early-apoptotic (Annexin V$^+$ 7AAD$^-$) and late-apoptotic/necrotic ones (Annexin V$^+$ 7AAD$^+$ or 7AAD$^+$ only) [210], [211]. The cells were stained for CD8, CD44 and CD62L expression and analysed for cell death type in the three CD8$^+$ T cell subsets identified by CD44 and CD62L expression. Upon splenocyte isolation, the cells were counted. The spleens of mice fed with Tam had fewer cells than the control group, which was not unexpected, as a Tam induced loss of cells in the spleen of R26Cre.ER^{T2} mice has already been described [208]. The percentages of the three CD8$^+$ T cell subsets were not significantly altered, although a minor reduction could be seen in the effector memory subset of Tam fed mice (Figure 32).

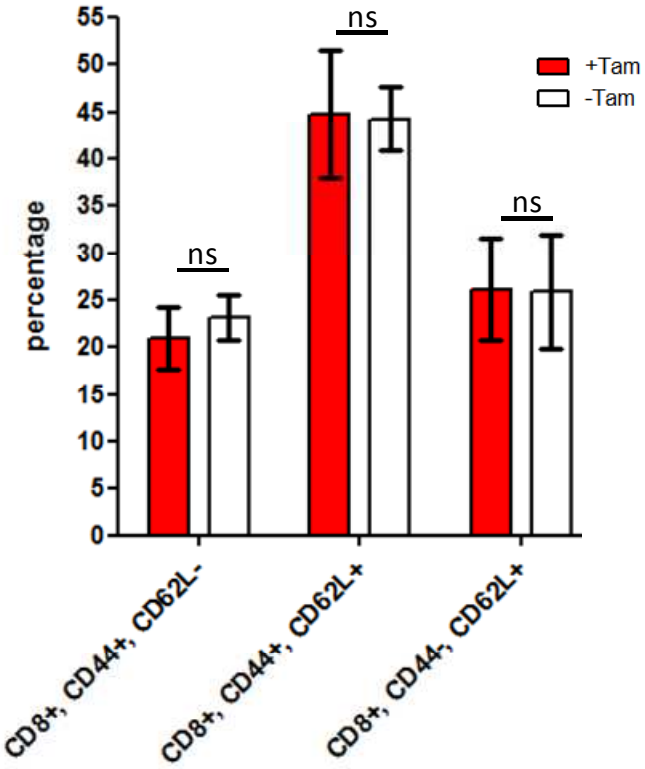

Figure 32 Effector memory T cells are not decreased after 2 weeks of Tamoxifen administration in R26Cre.ER^{T2}, infected with MCMV WT.

C57BL/6 R26Cre.ER^{T2} mice were i.p. infected with 10^6 PFU MCMV WT. 21 months later, mice were fed with pellets containing 400 mg/kg Tam for 2 weeks. 4x10^6 splenocytes were stained for CD8a, CD44 and CD62L expression. Pooled averages from 4 mice per group are shown. Error bars indicate SEM. Significance was assessed by Mann-Whitney U test (two-tailed) *$p < 0.05$.

The different T cell subsets were analysed with Annexin V and 7AAD for cell death. As can be seen in Figure 33, only the T_{EM} cells showed elevated percentages of late-apoptotic/necrotic cells. The fraction of apoptotic cells remained the same upon Tam treatment in the central memory and naive T cell compartment. Since the death rate of T_{CM} and naive T cells was not affected by Tam administration, the data argued that the reduction of the T_{EM} cell compartment (Figure 25 and Figure 31) was not caused by the loss of naive or T_{CM} cells replenishing the effector memory pool. In contrast, T_{EM} cells in the spleen were directly targeted upon Tam administration to the R26Cre.ER^{T2} mice, strengthening the evidence that elevated fractions of the inflationary CD8$^+$ T cells and the effector memory subset are maintained (at least in part) by cycling T_{EM} cells.

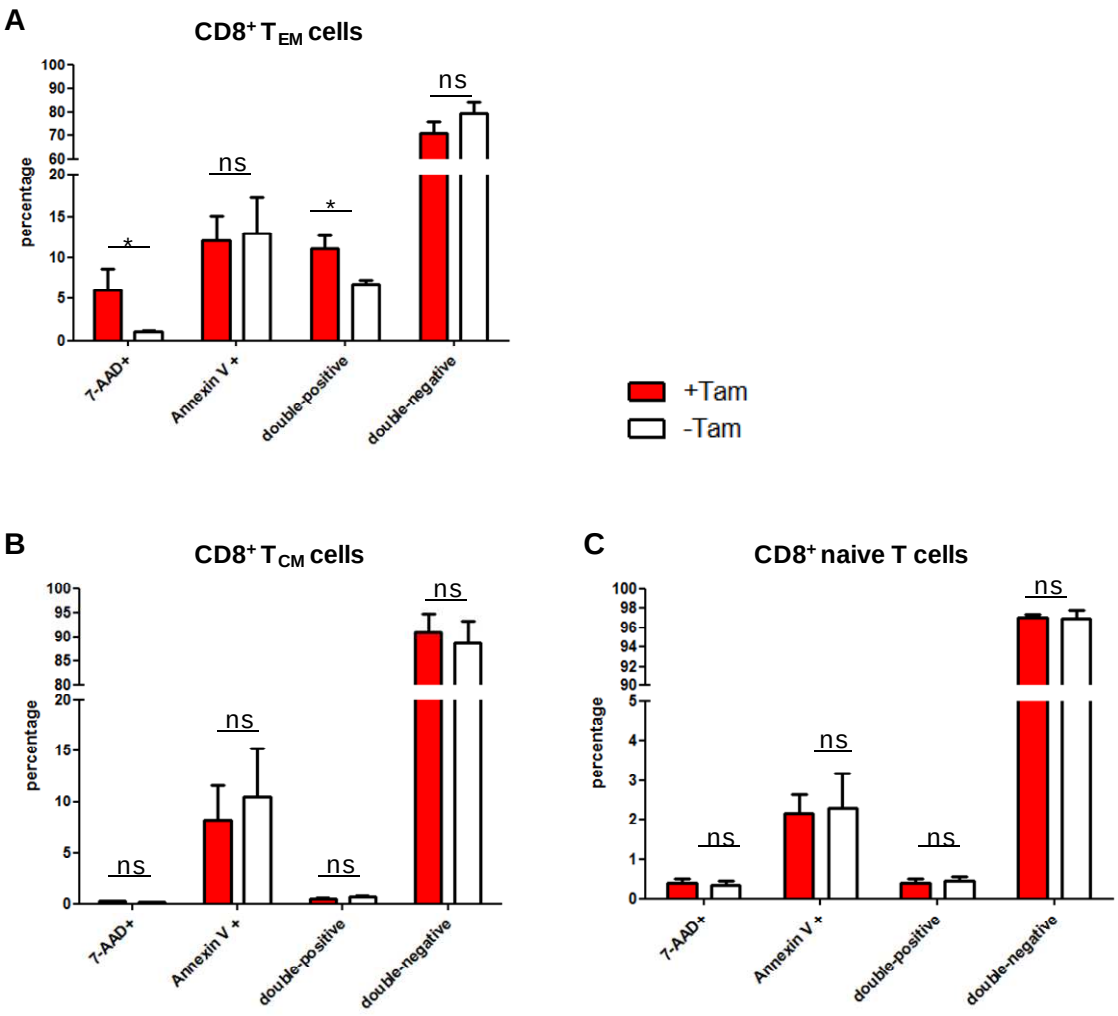

Figure 33 Activated Cre.ER^{T2} induce cell death only in the effector memory CD8$^+$ T cells.

C57BL/6 R26Cre.ER^{T2} mice were i.p. infected with 10^6 PFU MCMV. 21 months later, mice were fed with pellets containing 400 mg/kg Tam for 2 weeks. 4x10^6 splenocytes were stained for CD8a, CD44, CD62L, 7AAD and Annexin V. (**A**) CD8$^+$ T_{EM} cells (CD8$^+$ CD44$^+$ CD62L$^-$) were analysed with 7AAD and Annexin V. (**B**) CD8$^+$ T_{CM} cells (CD8$^+$ CD44$^+$ CD62L$^+$) were analysed with 7AAD and Annexin V. (**C**) The naive T cells (CD8$^+$ CD44$^-$ CD62L$^+$) were analysed with 7AAD and Annexin V. Pooled averages from 4 mice per group are shown. Error bars indicate SEM. Significance was assessed by Mann-Whitney U test (two-tailed) *$p < 0.05$.

4 Discussion

In HCMV seropositive individuals ~10% of the $CD4^+$ and $CD8^+$ T cells were found to be HCMV-specific [152]. The same phenomenon was observed also in mice where CMV-specific $CD8^+$ T cells with a T_{EM} phenotype inflate through the lifetime of latently infected hosts. This progressive accumulation of specific cytotoxic $CD8^+$ T_{EM} cells is termed memory inflation (MI) [148]. We showed recently that the absolute number of $CD8^+$ T cells is increased in MCMV-infected mice, but also that their composition is permanently altered, where the defining phenotype is a drastic increase of the proportion of T_{EM} cells [153].
To understand the dynamic of MI, two questions need to be addressed: From which T cell pool do the inflationary T_{EM} cells come and why do they accumulate over time?

Recent thymic emigrants in the naive T cell compartment are not likely contributors to MI, because the CMV specific $CD8^+$ T cells accumulate also in thymectomised mice [136]. Since CMV-specific T_{EM} cells have been described as short-lived cells, which do not proliferate extensively upon adoptive transfer, the T_{CM} cells have been suggested as a possible source of cells which replenish the T_{EM} cell pool [150]. MI also occurs and is maintained in mice whose APCs are unable to cross-present antigens to T cells in the latent phase of infection [93], but also in mice infected with a recombinant MCMV that is unable to reactivate [157]. It has therefore been proposed that local viral gene expression during latency might stimulate T_{CM} cells in the lymph nodes, resulting in their proliferation and differentiation into T_{EM} cells [159].

This work has aimed to study the effect of persistent viral gene expression on the maintenance of MI by means of a recombinant MCMV, allowing the conditional deletion of an essential and immunodominant genomic region.
The immediate-early genes *ie1/3* of MCMV were targeted for conditional deletion, because they are transcribed at low levels during viral latency and IE1- or IE3-specific T_{EM} cells accumulate over time of infection [147, 148]. Therefore, the *ie1/3* exons 1 to 5 were flanked by two *loxP* sites and transgenic mice expressing a Tam inducible Cre recombinase ($Cre.ER^{T2}$) were infected with the recombinant MCMV $IE1/3^{flox}$. Hence, the administration of Tam, allowed the selective targeting of the *loxP* sites at times of viral latency.

It was considered that the Cre recombinase may not easily reach its target and perform the recombination once the latent viral genome is highly associated with cellular repressors and deacetylated histones [74, 78]. While this condition prevents the RNA-Polymerase from binding to viral promoters, resulting in a transcriptionally inactive viral genome, the same chromatinisation may have also presented an obstacle for the binding of the Cre recombinase to the *loxP* sites and thus recombination. However, the presence of *ie1* and *ie3* transcripts during viral latency indicates that the *ie1/3* locus is not permanently silenced [53]. Studies from the Koszinowski group showed that the Cre recombinase can bind *loxP* sites in the viral genome and recombines up to 90% of viral genomes *in vivo* [169, 212]. The *in vitro* data from this study indicated that the Cre recombinase cannot prevent MCMV IE1/3^{flox} replication, but that it may be able to recombine viral genomes that are silenced by IFNβ. It is important to note that MCMV IE1/3^{flox} grew comparably to the WT virus in cells expressing Cre recombinase *in vitro*, probably indicating that the *ie* gene expression and viral DNA replication is much quicker than Cre-mediated recombination in most of the infected cells. On the other hand, when the MCMV IE1/3^{flox} or the MCMV WT replication and spread were inhibited by IFNβ, Cre had significantly more time to act on the viral genomes and IFNβ retraction at 7 dpi reactivated MCMV WT in 20% of the infected wells, whereas MCMV IE1/3^{flox} failed to reactivate (Figure 18). In sum, these results were consistent with a scenario where the Cre recombinase recombined the silenced *ie1/3* locus, resulting in an IE3 deficient MCMV, which could not grow any longer.

The selective pressure from the host immune system might cause mutations in the MCMV genome which are favourable for virus replication. This may also affect the exogenously inserted *loxP* sites. Therefore, it was important to check the modified region upon several replication cycles of the recombinant MCMV *in vivo*. For this, viral DNA was isolated from the mouse 3 weeks after infection to sequence the *loxP* sites and no mutations were found. In addition, the functionality of the *loxP* sites of the virus isolated from mice was analysed *in vitro* and no impairment was found (chapter 3.3). These data suggested that the *loxP* sequences are not affected during viral replication *in vivo* and that they would keep their functionality in the latent phase of infection, once the Cre recombinase would be induced to target them.

This study focused on the accumulation of CMV-specific $CD8^+$ T cells and in particular on the IE3-specific $CD8^+$ T cells recognising the immunodominant and inflationary peptide RALEYKNL (reviewed in [137] and [147]). The $CD8^+$ T cells of MCMV $IE1/3^{flox}$-infected R26Cre.ER^{T2} mice showed comparable accumulation from 60 dpi to those of mice infected with the MCMV WT (Figure 20). These data indicated that the insertion of the *loxP* sequences into the *ie1/3* locus and the presence of inactive Cre.ER^{T2} had not impaired the establishment of MI.

Tam was administered over a period of four weeks to R26Cre.ER^{T2} mice, starting at 120 dpi, once MCMV latency has already been established [52, 207]. This resulted in a shift in the T cell subsets within the $CD8^+$ T cells of MCMV $IE1/3^{flox}$-infected mice, but unexpectedly also of control mice infected with MCMV WT: The percentage of T_{EM} cells was decreased, the proportion of the T_{CM} cell subset was increased, whereas the percentage of naive T cells was not affected (Figure 23). The same phenomenon could be observed within the IE3-specific $CD8^+$ T cells, where the percentage decreased from 5 to 3 percent (Figure 22). The CMV-specific $CD8^+$ T cells with an inflationary phenotype are part of the T_{EM} cell subset. Since no decrease was noted in the T_{CM} and naive T cell subsets, it seemed as though only the CMV-specific T cells have been affected.

Tam is widely used in breast cancer therapy, where it blocks the oestrogen-receptor and thus decrease the risk of oestrogen-induced breast cancer return (reviewed in [213]). However, it can have severe side effects. Patients treated with Tam may have an increased risk of venous thrombosis, pulmonary emboli or uterine cancer [214, 215] and to develop non-alcoholic steatohepatitis [216]. In experiments with rats, even a carcinogenic effect on the liver could be observed [217]. It has also been shown that Tam inhibits MHC-II expression in DCs and therefore could have an influence on the stimulation of $CD4^+$ T cells [218], which in turn could have an influence on the MI of IE3-specific T cells [46]. However, this effect appeared entirely dependent on the presence of Tam together with Cre.ER^{T2}, because it did not occur in the absence of Tam or in mice lacking Cre.ER^{T2}. Experiments with another R26Cre.ER^{T2} positive mouse line and another recombinant MCMV presented the same Tam-induced effect on the frequency of T_{EM} cells (Figure 30 and Figure 31), but Tam had no effect on the numbers of IE3-specific T cells and T_{EM} cells in C57BL/6 mice infected with MCMV WT (Figure 26 - Figure 28).

Therefore, Tam appeared not to cause the decline of IE3-specific or T_{EM} $CD8^+$ T cells in the absence of Cre.ER^{T2}.

The Cre recombinase specifically recognises the *loxP* sites and recombines the DNA sequence next to it upon moderate expression such as under the control of the Rosa26 promoter. However, when it is expressed from a highly active promoter, the enzyme may bind to genomic sequences which resemble *loxP* sites and thus rise to chromosomal rearrangements [219]. The highly activated Tam-inducible variants are also assumed to act on the genomic DNA. It is assumed that the enzyme does not bind randomly in the genome, but rather at specific sites with conserved motifs. It is known that Cre can tolerate some mutations in the *loxP* sequence, and sequences that are almost identical in their sequence to the *loxP* sites have been found in the mammalian genome and termed *pseudo* or *cryptic loxP*-sites [220]. For some of these sites Cre-mediated recombination has already been proven [209]. As well as for the normal Cre recombinase, chromosomal abnormalities were detected for the Tam-inducible Cre.ER$^{T1/2}$ *in vitro* in MEFs and *in vivo* in bone marrow-derived cells [208, 221].

Higashi *et al*. described toxic effects of the highly activated Cre.ER^{T2} on embryonic tissue and hematopoietic cells [208]. In embryos, the activation of Cre.ER^{T2} resulted in haematological changes and lower liver and body weight. To analyse effects in the adult mice, their group focused on the bone marrow, the thymus and the spleen and described a decrease in cell number after Tam treatment in these organs. This effect was especially pronounced in the immature cells of the haematopoietic cell lineage, like erythroblasts or immature B cells and the CD4 CD8 double-positive T cells in the thymus. Likewise, the number of myeloid cells was decreased in the bone marrow and spleen, whereas the differentiation of bone marrow-derived progenitor cells into myeloid cells and their survival did not seem to be affected *in vitro*. Importantly, the number of proliferating cells was reduced in the thymus and the number of apoptotic cells was increased in the spleen, but the mature B cells and the cell density in the white pulp of the spleen were not affected. They concluded that activated Cre.ER^{T2} affects proliferating cells and increase apoptosis in immature cells of the hematopoietic cell lineage in primary lymphoid tissues as well as in the spleen.

This might imply that either immature haematopoietic cells or the highly proliferating ones (or cells that satisfy both criteria) are more sensitive to Cre-mediated toxicity. The reasons for that specific toxicity are unknown, but it might be due to cell type-specific gene expression and the associated epigenetic modifications.

The data from this work showed a decrease in T_{EM} cells that occurred only upon Tam application in mice expressing the Cre.ER^{T2}. The inactivated Cre.ER^{T2} did not affect the composition of the T cell subsets and the Tam alone resulted in no changes as well. This indicates that the Tam-activated Cre recombinase acted on mature CD8$^+$ T cells, which has not been described to date. The effect was reversible, as one month after completion of Tam treatment the percentages of T cell subsets recovered to levels seen in untreated mice.

Taking these results together, the conditional Cre recombinase expressed under a ubiquitous promoter cannot be used to analyse effects on MCMV-specific CD8$^+$ T cells. Although, the initial question could not be answered due to the limitations of the experimental model, the very same feature of the experimental system allowed the study of the origin of the CMV-specific CD8$^+$ T cells with inflationary phenotype during latency.

In theory, the T cell pool of MI could be maintained by long-term survival of cells in this compartment or by replenishing by the recruitment of naive T cells, the proliferation of T_{EM} cells and the proliferation and differentiation of T_{CM} cells. Naive T cells may be recruited to the MI pool, but their presence is not necessary to maintain the inflationary T cells [150, 158]. Snyder *et al*. showed that memory-inflated cells in the MCMV-specific T_{EM} cell subset are short-lived [150]. These cells have a half-life of ~ 45 - 60 days, so the MI pool would have to be constantly replenished by newly differentiated T_{EM} cells. Torti *et al*. demonstrated that the CD8$^+$ T cells specific for the inflationary epitope M38 are mainly composed of T_{CM} cells and to a lesser extent of T_{EM} cells in the lymph nodes and hypothesised that latently infected non-hematopoietic cells stimulate MCMV-specific T_{CM} cells in lymph nodes to proliferate and differentiate into T_{EM} cells, which migrate into the peripheral tissues and do not further proliferate [159]. They did not experimentally prove that latent MCMV is present in the LN, and they did not address the possibility that other organs (e.g. spleen) may be relevant for the maintenance of the pool of MCMV specific CD8$^+$ T cells. Others demonstrated that the spleen is a site of CMV latency [50, 52] and that splenic T_{EM} cells may proliferate in a latently infected mouse, although not as good as T_{CM} cells [150]. Hence, it is possible that both memory subsets may be involved in the production of MCMV-specific T_{EM} cells that migrate to the end organ [159]. On the other hand, little to no proliferation of MCMV-specific CD8$^+$ T cells takes place in the lung [159, 222], although this is a major site of virus recurrence [223], while T cell proliferation takes place in the lymph

nodes of the very same mice [159]. Taken together, the data indicate that the ability of inflationary T cells to proliferate may critically depend on the organ in which they reside, either due to the presence of antigen-presenting cells or the cytokine profile of the organ or both.

All three T cell subsets, naive T cells, T_{CM} and T_{EM} cells are found in the spleen (reviewed in [187, 188]). Therefore, it was feasible that the toxic effect of the activated Cre.ER^{T2} on metabolically active cells or the proliferating T cell subset may be identified in this organ.

Different splenic CD8$^+$ T cell subsets of latently infected R26Cre.ER^{T2} mice infected with MCMV WT for 21 month were analysed for apoptosis upon 2 weeks of Tam administration. Total splenocyte counts were lower than in the control group, in line with data from published literature [208]. The only T cell subset with significantly increased apoptosis after Tam treatment, were the T_{EM} cells. This implies that the CD8$^+$ T cells with an effector memory phenotype (CD44$^+$ CD62L$^-$) were the major target for the highly activated Cre.ER^{T2} in the spleen. In contrast the T_{CM} cells (CD44$^+$ CD62L$^+$) and the naive T cells (CD44$^-$ CD62L$^+$) were not affected by the toxic effect of Cre.ER^{T2}. These result differs from the results that one would expect from the model proposed by Snyder *et al*. and Torti *et al*., where such a decrease would rather have been expected in the pool of T_{CM} cells, as they appeared to have the largest ability to proliferate and to differentiate [150, 159].

The induction of apoptosis could be a response to either chromosomal abnormalities during the proliferation of the T_{EM} cells or during the differentiation from T_{CM} cells to T_{EM} cells. Moreover, the presence of T cell survival signals might play a role. The cytokines IL-7 and IL-15 are known to be involved in generating and maintenance of T_{CM} cells (reviewed in [224]). In the spleen, as well as in the lymph nodes, IL-7 is produced by splenic stromal cells, which are located in the T cell rich white pulp [225]. IL-15 is produced for example by professional APCs [226, 227]. Additionally, the cytokines IL-2, secreted by lymphocytes, and IL-12, secreted by APCs, are involved in T cell proliferation [228]. However, the survival of T_{EM} cells is not dependent on IL-7 and IL-15 as they have low or no expression of the IL-7 receptor CD127 or the IL-15 receptor CD122 [149]. Since the relative number of naive T cells and T_{CM} cells was not affected upon Tam administration in R26Cre.ER^{T2} mice and these cells did not show increased apoptosis, it is unlikely that the effects of Tam administration were due to effects involving IL-7 or IL-15 expression levels in the spleen. Potential indirect Cre toxicity on T_{EM} cells by a suppression of IL-2 or IL-12 cannot be formally excluded, as the level of

cytokines was not examined in the samples. IL-12 is, among others, secreted by cells of the myeloid cell lineage, especially by professional APCs [228]. It was shown that the absolute number of cells positive for the markers CD11b and Ly6G (myeloid cells) are decreased after R26Cre.ER^{T2} mice received Tam [208]. Whether these cells were dead or migrated out of the spleen had not been studied, but the differentiation and maintenance of CD11b cells *in vitro* was not impaired in the presence of Tam [208]. The lack of data about the cytokine profile of the spleen before and after the Tam administration in R26Cre.ER^{T2} mice allows the possibility that differences in IL-12 secretion affected the death rate of T_{EM} cells, although this would imply that IL-12 is necessary only for the survival of T_{EM} and not T_{CM} cells. In conclusion, the role of IL-12 in the Cre-mediated killing of T_{EM} cells may be addressed in future studies by addressing the in vivo proliferation of the various T cell subsets.

T_{EM} and T_{CM} cells differ in the expression of various surface molecules, but also in their functions and gene expression profiles. This difference could possibly lead to differences in genomic regions that would be accessible to the Cre recombinase in these cell types. Therefore, it is not possible to formally exclude the possibility that highly activated Cre recombinase may have access to *pseudo loxP* sites exclusively in T_{EM} cells. The same could have happened during the differentiation of a T_{CM} cell to a daughter T_{EM} cell. On the other hand, it is also possible that T_{EM} cells in the spleen are capable of proliferation that is comparable to or better than the T_{CM} cells and therefore would be more susceptible to Cre-mediated toxicity.

The initial question of this work could not be answered because the selected model system of Tam-induced Cre recombinase turned out to be unsuitable to study MI of $CD8^+$ T cells in an MCMV infection. However, there are other experimental mouse models that could be tested to study the influence of *ie1/3* expression on MI with the Cre/*loxP* system.

In one system, the expression of Cre recombinase can be controlled by the administration of Tetracycline or its derivate Doxycycline (Dox). In this model, the Cre recombinase is expressed under the control of a weak promoter. Between the enhancer and the promoter are binding sites (operator sequences or tetracycline-responsive elements (tetO)) for the reverse tetracycline-controlled transactivator (rtTA) which is also encoded in the mouse genome. The rtTA binds to the tetO elements only upon Dox administration and thereby activates the transcription of the Cre recombinase. (reviewed in [229])

However, this system has limitations, because even in the absence of Dox there is a basal expression of Cre recombinase.

Furthermore it may be possible to use mouse models with constitutive expression of active Cre recombinase. The CMV-Cre transgenic mice express the Cre recombinase ubiquitously under the control of the strong HCMV minimal promoter [230]. Here, the *ie1/3* region would be targeted by the Cre recombinase in all cells during the primary infection and viral latency, increasing the chance of recombination. However, this is also the disadvantage of this mouse line. The knock-out of the *ie1/3* region would be initiated immediately upon infection in all infected cells, which may diminish the latent load of the virus, in contrast to the system where the knock-out could be induced at a defined time point, once the virus is already latent.
Other interesting transgenic mouse lines are the mice which express the Cre recombinase in endothelial cells (ECs) only, under the control of the EC-specific Tie-2 promoter [231-233]. The Tie-2 promoter is active in all ECs during the embryogenesis, but also in the adult animal [231, 234]. These mouse lines would be particularly interesting, since ECs are proposed to be a site of latency for MCMV, which has already been proven for the LSECs [53]. This system also relies on the Cre recombinase that is constitutively active during primary infection, but the ECs play only a minor role in the reproduction and spread of MCMV in the primary phase of infection [235]. Using these mice it could be clarified, whether ECs are the only cell type in which the virus remains latent and further if the *ie1/3* gene expression in these cells is necessary for the induction or maintenance of MI.

The study of the interaction of CMV and its host remains an arduous task, since infection with CMV can affect every organ in the body, in both the acute and latent phase. In the organs and tissues all three $CD8^+$ T cell subsets have been identified, but their composition may vary. In the lungs high amounts of T_{EM} cells have been detected [236], whereas in the lymph nodes more naive T cells have been located [153], which is due to the cell composition and the cytokine profile within the organ. Published data indicated that the MCMV specific T_{CM} cells have high proliferative capacity in the lymph nodes [159], and thus should be affected by the Cre-mediated toxicity in R26Cre.ER^{T2} mice. Instead we observed significant cell death within the T_{EM} cell population, but no increase in apoptosis in T_{CM} and naive T cells upon Tam treatment in the spleen. Therefore, for a more conclusive statement about the proliferative capabilities of the different $CD8^+$ T cell subsets in the spleen, the cells would need to be directly tested for

their *in vivo* proliferation, for instance by assessing the expression of Ki-67 (reviewed in [237]), or other markers of cell cycling in the different subsets.

In conclusion, despite the limitations of the Tam-inducible Cre/*lox*P system that leads to the Cre-mediated toxicity on the $CD8^+$ T cells, the data from this work argue that MCMV infection of R26Cre.ER^{T2} mice presents a novel model to study the proliferative capacity of memory $CD8^+$ T cell subsets. Therefore, this system offers great potential as a tool to characterise the drivers and the mechanisms of the maintenance of the memory $CD8^+$ T cell compartment under homeostatic conditions.

5 References

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6 Appendix

6.1 Primer and Construct List

Table 2 List of Primers used in this study.

The Primers were designed with the software Vector NTI and ordered at Eurofins MWG Operon (Ebersfeld, DE).

denotation	sequence	size	gene/ plasmid
1	ATCATCCGTTGCATCTCGTTG	21-mer	MCMV *m54*
9	GTGTACTAGTGCTCTTCTTAGTTATGTATC TACATATTTTACAAAACAGGGTTCATCTTT AATACCACCTGTTGACAATTAATCATCGG CA	91-mer	*pGPS/galKn*
11	GTGTAAGCTTGCTCTTCCATCATGAGGAT GAGTCTGGGGAGTATGAGTCTGACTGCG AGTGATCTGTGCCAGTGTTACAACCAATT AACC	90-mer	*pGPS/galKn*
18	GGATTTATTTAATAGGGTTCGACATGAG	28-mer	MCMV *ie1/3*
19	TGAATCCTCTTCCCATCTATGAG	23-mer	MCMV *ie1/3*
20	GCCTGCATTACCGGTCGATGCAACGA	26-mer	*Cre.ER^{T2}*
21	GTGGCAGATGGCGCGGCAACACCATT	26-mer	*Cre.ER^{T2}*
43	GCTCTTCACCGAGCGGGTCCGATGCGCT TCAGTAGCCGGGGAAGGTCGCTCTTTCTT CCTGTTGACAATTAATCATCGGCA	81-mer	*pGPS/galKn*
45	GCTCTTCGTCGCAGTCTTCGGTCTGACCA CCGTAGAACGCAGAGCTCCTCGCTGCAG GCCAGTGTTACAACCAATTAACC	80-mer	*pGPS/galKn*
46	ACCAATATGGCGGCTGTTCC	20-mer	MCMV *ie1/3*
47	TGGCACGCATTCTATTGGCT	20-mer	MCMV *ie1/3*
97	CGCCAGTCTGTATCCGTCCAT	21-mer	MCMV *m54*
102	CAGCGCTCCTCCTGATAC	18-mer	*Rag2^{flox}*
103	TGCATTCCTAGAGCGTCCTT	20-mer	*Rag2^{flox}*

135	CTGTGGACAGAGGAGCCATAACTGC	25-mer	*Rosa26*
136	CCACCACTGGCTGGCTAAACTCT	23-mer	*Rosa26*
300	ACGACCAAGTGACAGCAATG	20-mer	*Cre*
301	CTCGACCAGTTTAGTTACCC	20-mer	*Cre*
351	GCCAGCTGAGACTTGGCATCCAGGAAAGGC	30-mer	MCMV *ie1/3*
352	ATGAGCTACGTAGGTGAGGCCATAGTGGCA	30-mer	MCMV *ie1/3*
353	ATCAATCAGCCATCAACTCTGCTACCAACA	30-mer	MCMV *ie1/3*
354	ATGGTGAAGCTATCAAAGATGTGCATCTCA	30-mer	MCMV *ie1/3*

Table 3 List of construct used in this study.

The constructs contain the *loxP* sequence and homology sequences to the site in the viral genome at which the *loxP* sequence was introduced. They were designed with the software Vector NTI and ordered at the listed company.

construct	**sequence**	**company**
loxP sequence at the 3' end of *ie3*	TTAATTAAAAGCTTAGTTAGTTAGTTAGTTATGTATCTACATATTTTACAAAACAGGGTTCATCTTTAATACCAATAACTTCGTATAATGTATGCTATACGAAGTTATACAGATCACTCGCAGTCAGACTCATACTCCCCAGACTCATCCTCATGATGGCTTATATCAAAGCTTGGCGCGCC	GENEART (Regensburg, DE)
loxP sequence into Exon 1 of *ie1/3*	TCCCAATAGAACCGAGCGGGTCCGATGCGCTTCAGTAGCCGGGGAAGGTCGCTCTTTCTTATAACTTCGTATAATGTATGCTATACGAAGTTATCTGCAGCGAGGAGCTCTGCGTTCTACGGTGGTCAGACCGAAGACTGCGACGGTACCGACG	Eurofins MWG Operon (Ebersfeld, DE)

6.2 PCR Mix and Programs for Viral Mutagenesis

6.2.1 MCMV *ie3* pA *galK-Kn*

To amplify the *galKn* cassette from the pGPS/galKn plasmid with primers having homologies to the 3' end of *ie1/3* the following mix was used:

50 µl in total:	1 µl	4 ng Plasmid-DNA
	10 µl	5x Puffer
	1 µl	dNTPs (2,5 mM each dNTP)
	1 µl	Primer 9 (10 pmol)
	1 µl	Primer 11 (10 pmol)
	5 U	*LongAmp* DNA Polymerase (NEB, MA, USA)
	Adjust to 50 µl	H_2O

The following PCR program was used:

1.	Lid	103℃
2.	Initial denaturing	94℃ for 5 min
3.	Denaturing	94℃ for 30 sec
4.	Annealing	64℃ for 30 sec
5.	Elongation	72℃ for 2 min
6.	Repeat	step 3. to 5. 18 times with reduction of annealing temperature for 1℃ every cycle
7.	Denaturing	94℃ for 30 sec
8.	Annealing	45℃ for 30 sec
9.	Elongation	72℃ for 2 min
10.	Repeat	step 7. – 9. 17 times
11.	Elongation	72℃ for 7 min
12.	Storage	at 4℃

6.2.2 MCMV *ie3* pA *loxP*

To amplify the *ie3* pA *loxP* construct from the plasmid send by GENEART the following mix was used:

50 µl in total:	1 µl	4 ng Plasmid-DNA
	10 µl	5x Puffer
	1 µl	dNTPs (2,5 mM each dNTP)
	1 µl	Primer 18 (10 pmol)
	1 µl	Primer 19 (10 pmol)
	5 U	*LongAmp* DNA Polymerase (NEB, MA, USA)
	Adjust to 50 µl	H_2O

The following PCR program was used:

1.	Lid	103℃
2.	Initial denaturing	94℃ for 5 min
3.	Denaturing	94℃ for 30 sec
4.	Annealing	60℃ for 30 sec
5.	Elongation	72℃ for 1 min
6.	Repeat	step 3. to 5. 18 times with reduction of annealing temperature for 1℃ every cycle
7.	Denaturing	94℃ for 30 sec
8.	Annealing	45℃ for 30 sec
9.	Elongation	72℃ for 2 min
10.	Repeat	step 7. – 9. 25 times
11.	Elongation	72℃ for 7 min
12.	Storage	at 4℃

6.2.3 MCMV *ie1/3* Exon1 *galK-Kn*

To amplify the *galKn* cassette from the pGPS/galKn plasmid with primers having homologies to the 5' end of *ie1/3* the following mix was used:

50 µl in total:	1 µl	4 ng Plasmid-DNA
	10 µl	5x Puffer
	1 µl	dNTPs (2,5 mM each dNTP)
	1 µl	Primer 43 (10 pmol)
	1 µl	Primer 45 (10 pmol)
	5 U	*LongAmp* DNA Polymerase (NEB, MA, USA)
	Adjust to 50 µl	H_2O

The following PCR program was used:

1.	Lid	103℃
2.	Initial denaturing	94℃ for 5 min
3.	Denaturing	94℃ for 30 sec
4.	Annealing	64℃ for 30 sec
5.	Elongation	72℃ for 2 min
6.	Repeat	step 3. to 5. 18 times with reduction of annealing temperature for 1℃ every cycle
7.	Denaturing	94℃ for 30 sec
8.	Annealing	45℃ for 30 sec
9.	Elongation	72℃ for 2 min
10.	Repeat	step 7. – 9. 17 times
11.	Elongation	72℃ for 7 min
12.	Storage	at 4℃

6.2.4 MCMV *ie1/3* Exon1 *loxP*

To amplify the *ie1/3* Exon1 *loxP* construct from the plasmid send by Eurofin MWG Operon the following mix was used:

50 µl in total:	1 µl	4 ng Plasmid-DNA
	10 µl	5x Puffer
	1 µl	dNTPs (2,5 mM each dNTP)
	1 µl	Primer 46 (10 pmol)
	1 µl	Primer 47 (10 pmol)
	5 U	*LongAmp* DNA Polymerase (NEB, MA, USA)
	Adjust to 50 µl	H_2O

The following PCR program was used:

1.	Lid	103℃
2.	Initial denaturing	94℃ for 5 min
3.	Denaturing	94℃ for 30 sec
4.	Annealing	60℃ for 30 sec
5.	Elongation	72℃ for 1 min
6.	Repeat	step 3. to 5. 18 times with reduction of annealing temperature for 1℃ every cycle
7.	Denaturing	94℃ for 30 sec
8.	Annealing	45℃ for 30 sec
9.	Elongation	72℃ for 2 min
10.	Repeat	step 7. – 9. 25 times
11.	Elongation	72℃ for 7 min
12.	Storage	at 4℃

6.3 PCR Mix and Programs to Detect Viral DNA

6.3.1 MCMV *ie3* pA *loxP*

To amplify the *ie3* pA *loxP* site from MCMV BAC or cell culture SN the following mix was used:

25 µl in total:	1 µl/0,5 µl	SN/ MCMV BAC
	12,5 µl	2x MangoMix (Bioline)
	1 µl	Primer 18 (10 pmol)
	1 µl	Primer 19 (10 pmol)
	Adjust to 25 µl	H_2O

The following PCR program was used:

1.	Lid	103℃
2.	Initial denaturing	94℃ for 5 min
3.	Denaturing	94℃ for 30 sec
4.	Annealing	60℃ for 30 sec
5.	Elongation	72℃ for 1 min
6.	Repeat	step 3. to 5. 18 times with reduction of annealing temperature for 1℃ every cycle
7.	Denaturing	94℃ for 30 sec
8.	Annealing	45℃ for 30 sec
9.	Elongation	72℃ for 2 min
10.	Repeat	step 7. – 9. 25 times
11.	Elongation	72℃ for 7 min
12.	Storage	at 4℃

6.3.2 MCMV *ie1/3* Exon1 *loxP*

To amplify the *ie1/3* Exon1 *loxP* site from MCMV BAC or cell culture SN the following mix was used:

25 µl in total:	1 µl/0,5 µl	SN/ MCMV BAC
	12,5 µl	2x MangoMix (Bioline)
	1 µl	Primer 46 (10 pmol)
	1 µl	Primer 47 (10 pmol)
	Adjust to 25 µl	H_2O

The following PCR program was used: as described above in MCMV *ie3* pA *loxP*.

6.3.3 MCMV *ie1/3* knock-out

To amplify the *ie1/3* knock-out from cell culture SN the following mix was used:

25 µl in total:	1 µl	SN
	12,5 µl	2x MangoMix (Bioline)
	1 µl	Primer 18 (10 pmol)
	1 µl	Primer 47 (10 pmol)
	Adjust to 25 µl	H_2O

The following PCR program was used: as described above in MCMV *ie3* pA *loxP*.

6.4 PCR Mix and Programs to Genotype Transgenic Mice

6.4.1 C57BL/6 R26Cre.ER^{T2} Part 1

To check for presence of Cre.ER^{T2} (Primer are binding in the gene Cre.ER^{T2}) the following mix was used:

25 µl in total:	1 µl	Genomic DNA
	12,5 µl	2x MangoMix (Bioline)
	1 µl	Primer 20 (10 pmol)
	1 µl	Primer 21 (10 pmol)
	Adjust to 25 µl	H_2O

The following PCR program was used:

1.	Lid	103℃
2.	Initial denaturing	94℃ for 5 min
3.	Denaturing	94℃ for 30 sec
4.	Annealing	67℃ for 30 sec
5.	Elongation	72℃ for 2 min
6.	Repeat	step 3. – 5. 35 times
7.	Elongation	72℃ for 7 min
8.	Storage	at 4℃

6.4.2 C57BL/6 R26Cre.ER^{T2} Part 2

To check if mice were homozygous or heterozygous for the gene Cre.ER^{T2} (Primer binds in the wild type gene Rosa26) the following mix was used:

25 µl in total:	1 µl	Genomic DNA
	12,5 µl	2x MangoMix (Bioline)
	1 µl	Primer 135 (10 pmol)
	1 µl	Primer 136 (10 pmol)
	Adjust to 25 µl	H_2O

The following PCR program was used:

1.	Lid	103℃
2.	Initial denaturing	94℃ for 5 min
3.	Denaturing	94℃ for 30 sec
4.	Annealing	61℃ for 30 sec
5.	Elongation	72℃ for 2 min
6.	Repeat	step 3. To 5. 15 times
7.	Denaturing	94℃ for 30 sec
8.	Annealing	58℃ for 30 sec
9.	Elongation	72℃ for 2 min
10.	Repeat	step 7. to 9. 15 times
11.	Denaturing	94℃ for 30 sec
12.	Annealing	55℃ for 30 sec
13.	Elongation	72℃ for 2 min
14.	Repeat	step 11. – 13. 15 times
15.	Elongation	72℃ for 7 min
16.	Storage	at 4℃

6.4.3 C57BL/6 Rag2^{flox}xR26Cre.ER^{T2} Part 1 and 2

To check for presence of Cre.ER^{T2} (Primer are binding in the gene Cre.ER^{T2}) the following mix was used: as described above for C57BL/6 R26Cre.ER^{T2} Part 1 and 2

6.4.4 C57BL/6 Rag2^{flox}xR26Cre.ER^{T2} Part 3

To check if mice were homozygous or heterozygous for the *loxP* sequences flanking the gene Rag2 the following mix was used:

25 µl in total:	1 µl	Genomic DNA
	12,5 µl	2x MangoMix (Bioline)
	1 µl	Primer 102 (10 pmol)
	1 µl	Primer 103 (10 pmol)
	Adjust to 25 µl	H_2O

The following PCR program was used:

1.	Lid	103℃
2.	Initial denaturing	94℃ for 5 min
3.	Denaturing	94℃ for 30 sec
4.	Annealing	55℃ for 30 sec
5.	Elongation	72℃ for 1 min
6.	Repeat	step 3. – 5. 35 times
7.	Elongation	72℃ for 7 min
8.	Storage	at 4℃

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